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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Variants of the gamma chain of AMPK, DNA sequences encoding the same, and uses thereof

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VARIANTS OF THE GAMMA CHAIN OF AMPK, DNA SEQUENCES ENCODING THE SAME, AND USES THEREOF.

The present invention relates to new variants of the γ chain of AMP-activated protein kinase (AMPK), to genes encoding said variants and to uses thereof.

AMPK has a key role in regulating the energy metabolism in the eukaryotic cell (HARDIE et al., Annu. Rev. Biochem., 67, 821-855, 1998; KEMP et al., TIBS, 24, 22-25, 1999). Mammalian AMPK is a heterotrimeric complex comprising a catalytic α subunit and two non-catalytic β and γ subunits that regulate the activity of the α subunit. The yeast homologue (denoted SNF1) of this enzyme complex is well characterised; it comprises a catalytic chain (Snf1) corresponding to the mammalian α subunit, and regulatory subunits: Sip1, Sip2 and Gal83 correspond to the mammalian β subunit, and Snf4 correspond to the mammalian γ subunit. Sequence data show that AMPK homologues exist also in *Caenorhabditis elegans* and *Drosophila*.

It has been observed that mutations in yeast *SNF1* and *SNF4* cause defects in the transcription of glucose-repressed genes, sporulation, thermotolerance, peroxisome biogenesis, and glycogen storage.

In the mammalian cells, AMPK has been proposed to act as a "fuel gauge". It is activated by an increase in the AMP:ATP ratio, resulting from cellular stresses such as heat shock and depletion of glucose and ATP. Activated AMPK turns on ATP-producing pathways (e.g. fatty acid oxidisation) and inhibits ATP-consuming pathways (e.g. fatty acid and cholesterol synthesis), through phosphorylation of the enzymes acetyl-CoA carboxylase and hydroxymethylglutaryl-CoA (HMG-CoA) reductase. It has also been reported to inactivate *in vitro* glycogen synthase, the key regulatory enzyme of glycogen synthesis, by phosphorylation (HARDIE et al.,

1998, *supra*); however, whether glycogen synthase is a physiological target of AMPK *in vivo* remained unclear.

Several isoforms of the three different AMPK subunits are present in mammals. In humans, *PRKAA1* on human chromosome (HSA) 5p12 and *PRKAA2* on HSA1p31 respectively encode isoforms α1 and α2 of the α subunit, *PRKAB1* on HSA12q24.1 and *PRKAB2* (not yet mapped) respectively encode isoforms β1 and β2 of the β subunit, and *PRKAG1* on HSA12q13.1 and *PRKAG2* on HSA7q35-q36 respectively encode isoforms γ1 and γ2 of the γ subunit (OMIM database, http://www.ncbi.nlm.nih.gov/omim/, July 1999). HARDIE et al., [1998, *supra*] also mention the existence of a third isoform (γ3) of the γ subunit of AMPK but do not provide any information about it. Analysis of the sequences of these γ subunits shows that they are essentially composed of four cystathione β synthase (CBS) domains whose function is unknown. No phenotypic effect resulting from a mutation in either of the AMPK subunits has yet been documented.

On the other hand, it has been observed that most Hampshire pigs have a high intramuscular glycogen concentration. In these pigs, glycogenolysis which occurs after slaughtering leads to an important decrease of the pH, resulting in acid meat having a reduced water-holding capacity and giving a reduced yield of cured cooked ham.

The locus (named *RN*) associated with high muscular content of glycogen was first identified by family segregation analysis of phenotypic data from Hampshire pigs (LE ROY *et al.*, Genet. Res., 55, 33-40, 1990). A fully dominant allele, *RN*$^-$, correlated with high glycogen content occurs at a high frequency in most Hampshire populations while pigs from other breeds are assumed to be homozygous for the normal, recessive *rn*$^+$ allele. Subsequent studies showed that *RN*$^-$ carriers have a large increase (about 70%) of glycogen in skeletal muscle

but not in liver (MONIN *et al.*, in 38th *ICoMST*, Clermont-Ferrand, FRANCE, 1992).

The large difference in glycogen content between *RN*⁻ and rn⁺ pigs leads to marked differences in meat quality and technological yield (ENFÄLT *et al.*, J. Anim. Sci., 75, 2924-2935, 1997). The *RN*⁻ allele is therefore of considerable economical significance in the pig industry and most breeding companies would like to reduce or eliminate this dominant mutation.

The RN phenotype can be determined by measuring the glycolytic potential in muscle biopsies from live animals, or after slaughter (MONIN *et al.*, Meat Science, 13, 49-63, 1985). However, this method has severe limitations for application in practical breeding programs. The accuracy of the test is not 100%: as there is some overlap in the phenotypic distribution of *RN*⁻ and rn⁺, the test is not able to distinguish *RN*⁻/*RN*⁻ homozygotes and *RN*⁻/*rn*⁺ heterozygotes. Further, the sampling of muscle biopsies on live animals is invasive and costly.

Thus, there is a strong need for the development of a simple diagnostic DNA test for the *RN* locus. Moreover, the dramatic phenotypic effect of the *RN* gene in pigs implies that this gene has an important role in the regulation of carbohydrate metabolism in skeletal muscle in other vertebrates, in particular mammals.

Skeletal muscle and liver are the two major reservoirs of glycogen in mammals and the observation of an increased muscular glycogen while liver glycogen is normal suggests that the RN⁻ phenotype maybe due to a mutation in a gene expressed in muscle but not in liver. The inventors have previously reported that the *RN* gene is located on pig chromosome 15 (MILAN *et al.*, Mamm. Genome, 7, 47-51, 1996; MARIANI *et al.*, Mamm. Genome, 7, 52-54, 1996; LOOFT *et al.*, Genetics Selection Evolution, 28, 437-442, 1996). They have now discovered that the *RN*⁻

allele is associated with a non-conservative mutation in a gene encoding a new muscle-specific isoform of the AMP-activated protein kinase (AMPK) γ chain.

The various aspects of the present invention are based upon the discovery and characterisation of this mutation and the identification and isolation of the mutant gene.

According to the invention it is shown that a mutation in a γ chain of AMPK results in an altered regulation of carbohydrate metabolism, demonstrating that AMPK is an essential component of said metabolism. It is also provided a nucleic acid sequence encoding a muscle-specific isoform of the γ chain of AMPK. Thus it is provided means to regulate carbohydrate metabolism, more specifically to detect and/or correct potential or actual dysfunctions of the regulation of carbohydrate metabolism, in particular in skeletal muscle.

The invention provides a polypeptide comprising an amino acid sequence having at least 70% identity or at least 85% similarity, preferably 80% identity or at least 90% similarity, more preferably at least 90% identity or at least 95% similarity, and still more preferably at least 95% identity or at least 99% similarity, with the polypeptide SEQ ID NO: 2. The invention also provides an isolated nucleic acid sequence encoding said polypeptide, as well as the complement of said nucleic acid sequence.

Said polypeptide represents a new muscle-specific isoform of the γ chain of AMPK, and will also be hereinafter referred as Prkag3; the gene encoding said polypeptide will also be hereinafter referred as *PRKAG3*.

"Identity" of a sequence with a reference sequence refers to the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residues positions. A polypeptide having an amino acid sequence having at least X% identity

with a reference sequence is defined herein as a polypeptide whose sequence may include up to 100-X amino acid alterations per each 100 amino acids of the reference amino acid sequence. Amino acids alterations include deletion, substitution or insertion of consecutive or scattered amino acid residues in the reference sequence.

"Similarity" of a sequence with a reference sequence refers to the percent of residues that are the same or only differ by conservative amino acid substitutions when the two sequences are aligned for maximum correspondence between residues positions. A conservative amino acid substitution is defined as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge or polarity), which generally does not change the functional properties of the protein. A polypeptide having an amino acid sequence having at least X% similarity with a reference sequence is defined herein as a polypeptide whose sequence may include up to (100-X) non-conservative amino acid alterations per each 100 amino acids of the reference amino acid sequence. Non-conservative amino acids alterations include deletion, insertion, or non-conservative substitution of consecutive or scattered amino acid residues in the reference sequence.

For instance, searching the "GenBank nr" database using BLASTp (ALTSCHUL *et al.*, Nucleic Acids Res., 25, 3389-3402, 1997) with default settings and the whole sequence SEQ ID NO: 2 as a query, the higher percents of identity or similarity with SEQ ID NO: 2 were found for:

- γ1 subunit of human AMPK: 65% identity or 82% similarity (score: 399);
- γ1 subunit of rat AMPK: 65% identity or 82% similarity (score: 399);

- γ1 subunit of murine AMPK: 64% identity or 80% similarity (score: 390);
- γ subunit of Drosophila AMPK: 53% identity or 75% similarity (score: 332);
- Yeast Snf4: 33% identity or 56% similarity (score: 173).

Polypeptides of the invention include for instance any polypeptide (whether natural, synthetic, semi-synthetic, or recombinant) from any vertebrate species, more specifically from birds, such as poultry, or mammals, including bovine, ovine, porcine, murine, equine, and human, and comprising, or consisting of, the amino acid sequence of either:

- a functional Prkag3; or
- a functionally altered mutant of Prkag3.

"Functional" refers to a protein having a normal biological activity. Such a protein may comprise silent mutations inducing no substantial change in its activity, and having no noticeable phenotypic effects. Non limitative examples of functional Prkag3 are:

- a porcine Prkag3 comprising at least the sequence represented in the enclosed sequence listing under SEQ ID NO: 2;
- a human Prkag3 comprising at least the sequence represented in the enclosed sequence listing under SEQ ID NO: 4;
- a V40I variant of Prkag3 resulting from the substitution of a valine residue in position 40 of SEQ ID NO: 2 or SEQ ID NO: 4 by an isoleucine residue.

The invention also includes splice variants of Prkag3: for instance, the nucleotide sequence and amino-acid sequence of 2 splice variants of porcine Prkag3 are represented in figure 7 (7a: nucleotide sequence; 7b: amino-acid sequence) and 8 (8a: nucleotide sequence; 8b: amino-acid sequence).

A "functionally altered mutant" of a protein comprises one or several mutations inducing a substantial change in its activity. Such mutations include in particular deletions, insertions, or non-conservative substitutions of amino acid residues in a domain essential for the biological activity of said protein. They may result for instance in a partial or total loss of activity, or conversely in an increase of activity, or in an impairment of the response to regulatory effectors. A non-limitative example of a functionally altered mutant of Prkag3 is the R41Q variant resulting from the non-conservative substitution of an arginine residue by a glutamine residue in position 41. This non-conservative substitution occurs inside a portion of the first CBS domain that is highly conserved between Prkag3 and the previously known isoforms of the γ subunit of AMPK.

Residue numbers for Prkag3 refer to the amino acid numbering of SEQ ID NO: 2 or SEQ ID NO: 4. Alignment of human and porcine Prkag3 sequences with previously known γ1 and γ2 isoforms is shown in Figure 3.

The invention also provides mutants of Prkag3 which may for instance be obtained by deletion of part of a Prkag3 polypeptide. Said mutants are generally functionally altered. They may have an identity with the overall Prkag3 sequence lower than 70%. However, the identity of the non-deleted sequences of said mutants, when aligned with the corresponding Prkag3 sequences should remain higher than 70%. Said mutants may for instance result from the expression of nucleic acid sequences obtained by deletion or insertion of a nucleic acid segment, or by a punctual mutation introducing a nonsense codon, in a nucleic acid sequence encoding a functional Prkag3.

The invention also provides a functionally altered mutant of a γ subunit of AMPK, wherein said mutant comprises at least one mutation responsible for said

functional alteration located within the first CBS domain, and preferably within the region thereof aligned with the region spanning from residue 30 to residue 50 of SEQ ID NO:2. Said mutation may result from the insertion, deletion, and/or non-conservative substitution of one amino-acid or of several amino-acids, adjacent or not. More preferably the mutation is located within the region aligned with the region spanning from residue 35 to residue 45 of SEQ ID NO:2, for instance within the region spanning from residue 65 to residue 75 of the γ1 isoform. According to a particular embodiment, said mutation is a non-conservative substitution, preferably a R→Q substitution. Advantageously, the mutation is located at a residue corresponding to residue 41 of SEQ ID NO:2, for instance at residue 70 of the γ1 isoform.

The invention also provides a heterotrimeric AMPK wherein the γ subunit consists of a polypeptide of the invention.

The invention also provides isolated nucleic acid sequences encoding any of the above-defined functional or functionally altered Prkag3 or functionally altered mutants of a γ subunit of AMPK, and nucleic acid sequences complementary of any one of these nucleic acid sequences.

This includes particularly any isolated nucleic acid having the sequence of any of the naturally occurring alleles of a *PRKAG3* gene, as well as any isolated nucleic acid having the sequence of an artificial mutant of a *PRKAG3* gene, provided that said nucleic acid does not consist of the EST GENBANK AA178898.

This also includes any isolated nucleic acid having the sequence of a natural or artificial mutant of a *PRKAG1* or a *PRKAG2* gene, wherein said mutant encodes a functionally altered γ1 or γ2 subunit of AMPK as defined above.

Nucleic acid sequences of the invention may be obtained by the well-known methods of recombinant DNA technology and/or of chemical DNA synthesis. These methods also allow to introduce the desired mutations in a naturally occurring DNA sequence.

Examples of nucleic acid sequences encoding naturally occurring alleles of a *PRKAG3* gene are SEQ ID NO: 1, which encodes a naturally occurring allele of the porcine gene and SEQ ID NO: 3, which encodes a naturally occurring allele of the human gene. They may be used to generate probes allowing the isolation of *PRKAG3* from other species or of other allelic forms of *PRKAG3* from a same species, by screening a library of genomic DNA or of cDNA.

The invention also includes genomic DNA sequences from any vertebrate species, more specifically from birds, such as poultry, or mammals, including in particular bovine, ovine, porcine, murine, equine, and human, comprising at least a portion of a nucleic acid sequence encoding a polypeptide of the invention, preferably a portion of a *PRKAG3* gene, and up to 500 kb, preferably up to 100 kb of a 3' and/or of a 5' adjacent genomic sequence.

Such genomic DNA sequences may be obtained by methods known in the art, for instance by extension of a nucleic acid sequence encoding a polypeptide of the invention, employing a method such as restriction-site PCR (SARKAR *et al.*, PCR Methods Applic., 2, 318-322, 1993), inverse PCR (TRIGLIA *et al.*, Nucleic Acids Res., 16, 8186, 1988) using divergent primers based on a Prkag3 coding region, capture PCR (LAGERSTROM *et al.*, PCR Methods Applic., 1, 111-119, 1991), or the like.

The invention also includes specific fragments of a nucleic acid sequence encoding a polypeptide of the invention, or of a genomic DNA sequence of the invention as well as nucleic acid fragments specifically

hybridising therewith. Preferably these fragments are at least 15bp long, more preferably at least 20bp long.

"Specific fragments" refers to nucleotidic sequences that are found only in the nucleic acids sequences encoding a polypeptide of the invention, and are not found in nucleic acids sequences encoding related polypeptides of the prior art. This excludes the nucleic acid fragments that consist of a sequence shared with one of the known *PRKAG1* or *PRKAG2* genes.

"Specifically hybridising fragments" refers to nucleic acid fragments which can hybridise, under stringent conditions, only with nucleic acid sequences encoding a polypeptide of the invention, without hybridising with nucleic acid sequences encoding related polypeptides of the prior art. This excludes the nucleic acid fragments that consist of the complement of a sequence shared with one of the known *PRKAG1* or *PRKAG2* genes.

Nucleic acid fragments that consist of the EST GENBANK AA178898 or the complement thereof are also excluded.

Said specific or specifically hybridising nucleic acid fragments may for example be used as primers or probes or for detecting and/or amplifying a nucleic acid sequence encoding a polypeptide of the invention. The invention encompasses set of primers comprising at least one primer consisting of a specific or specifically hybridising nucleic acid fragment as defined above.

The invention also provides recombinant vectors comprising a nucleic acid sequence encoding a polypeptide of the invention. Vectors of the invention are preferably expression vectors, wherein a sequence encoding a polypeptide of the invention is placed under control of appropriate transcriptional and translational control elements. These vectors may be obtained and

introduced in a host cell by the well-known recombinant DNA and genetic engineering techniques.

The invention also comprises a prokaryotic or eukaryotic host cell transformed by a vector of the invention, preferably an expression vector.

A polypeptide of the invention may be obtained by culturing the host cell containing an expression vector comprising a nucleic acid sequence encoding said polypeptide, under conditions suitable for the expression of the polypeptide, and recovering the polypeptide from the host cell culture.

A heterotrimeric AMPK wherein the γ subunit consists of a polypeptide of the invention may be obtained by expressing, together or separately, a nucleic acid sequence encoding a polypeptide of the invention, a nucleic acid sequence encoding an α subunit, and a nucleic acid sequence encoding a β subunit, and reconstituting the heterotrimer.

The polypeptides thus obtained, or immunogenic fragments thereof may be used to prepare antibodies, employing methods well known in the art. Antibodies directed against the whole Prkag3 polypeptide and able to recognise any variant thereof may thus be obtained. Antibodies directed against a specific epitope of a particular variant (functional or not) of Prkag3 or antibodies directed against a specific epitope of a functionally altered mutant having a mutation in the first CBS domain of a γ subunit of AMPK, and able to recognise said variant or functionally altered mutant may also be obtained.

As shown herein, mutations in a γ subunit of AMPK, and particularly mutations in the first CBS domain of a γ subunit of AMPK are likely to cause disorders in the energy metabolism (e.g. diabetes, obesity) in vertebrates, including humans. Further, mutations in the first CBS domain or other parts of the *PRKAG3* gene are

likely to cause disorders in the muscular metabolism leading to diseases such as myopathy, diabetes and cardiovascular diseases.

The present invention provides means for detecting and correcting said disorders.

More specifically, the present invention is directed to methods that utilise the nucleic acid sequences and/or polypeptidic sequences of the invention for the diagnostic evaluation, genetic testing and prognosis of a metabolic disorder.

For example, the invention provides methods for diagnosing of metabolic disorders, more specifically carbohydrate metabolism disorders, and preferably disorders correlated with an altered, in particular an excessive, glycogen accumulation in the cells, resulting from a mutation in a gene encoding a γ subunit of AMPK, wherein said methods comprise detecting and/or measuring the expression of a functionally altered *PRKAG3* gene, or of a functionally altered mutant of a γ subunit of AMPK having a mutation within the first CBS domain in a nucleic acid sample obtained from a vertebrate, or detecting a mutation in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK in the genome of a vertebrate suspected of having such a disorder.

According to a preferred embodiment of the invention, the disorder is correlated with an altered, in particular an excessive, glycogen accumulation in the muscular cells and results from the expression of a functionally altered *PRKAG3* gene.

The expression of a functionally altered Prkag3, or of a functionally altered mutant of a γ subunit of AMPK having a mutation within the first CBS domain may be detected or measured using either polyclonal or monoclonal antibodies specific for the functionally altered polypeptides of the invention, as defined above.

Appropriate methods are known in the art. They include for instance enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS).

The nucleotide sequences of the invention may be used for detecting mutations in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK, by detection of differences in gene sequences or in adjacent sequences between normal, carrier, or affected individuals.

The invention provides a process for detecting a mutation in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK wherein said process comprises:

- obtaining a nucleic acid sample from a vertebrate;
- checking the presence in said nucleic acid sample of a nucleic acid sequence encoding a mutant Prkag3, or a mutant of a γ subunit of AMPK having a mutation within the first CBS domain, as defined above.

According to a preferred embodiment of the invention there is provided a method for detecting a nucleic acid sequence comprising a mutation in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK wherein said process comprises:

- obtaining a nucleic acid sample from a vertebrate;
- contacting said nucleic acid sample with a nucleic acid probe obtained from a nucleic acid of the invention and spanning said mutation, under conditions of specific hybridisation between said probe and the mutant sequence to be detected;
- detecting the hybridisation complex.

Preferably, the process of the invention further comprises, prior to hybridisation, PCR amplification from the nucleic acid sample, of a sequence comprising at least the portion of the *PRKAG3* sequence or

of the sequence encoding the first CBS domain of the γ subunit of AMPK wherein the mutation is to be detected.

Methods allowing the specific hybridisation of a probe only with a perfectly matching complementary sequence, and useful for the detection of punctual mutations are known in the art. They include for instance Allele Specific PCR (GIBBS, Nucleic Acid Res., 17, 2427-2448, 1989), Allele Specific Oligonucleotide Screening (SAIKI *et al.*, Nature, 324, 163-166, 1986), and the like.

A mutation in the *PRKAG3* gene may also be detected through detection of polymorphic markers closely linked to said mutation.

The invention also provides means for identifying said polymorphic markers, and more specifically polymorphic markers comprised within a genomic DNA sequence comprising at least a portion of a *PRKAG3* gene, and up to 500 kb, preferably 300 kb, more preferably up to 100 kb of a 3′ and/or of a 5′ adjacent sequence.

Said polymorphic markers may be obtained for instance, by screening a genomic DNA library from a vertebrate with a probe specific for the *PRKAG3* gene, in order to select clones comprising said nucleic acid sequence and flanking chromosomal sequences, and identifying a polymorphic marker in said flanking chromosomal sequences. The allele(s) of a polymorphic marker associated with a given mutant allele of the *PRKAG3* gene may also easily be identified by use of a genomic DNA library from an individual wherein the presence of said mutant allele has previously been detected by hybridisation with a nucleic acid probe of the invention.

Polymorphic markers include for instance, single nucleotide polymorphisms (SNP), microsatellites, insertion/deletion polymorphism and restriction fragment length polymorphism (RFLP). These polymorphic markers may

be identified by comparison of sequences flanking the *PRKAG3* gene obtained from several individuals. Microsatellites may also be identified by hybridisation with a nucleic acid probe specific of known microsatellite motifs.

Once a polymorphic marker has been identified, a DNA segment spanning the polymorphic locus may be sequenced and a set of primers allowing amplification of said DNA segment may be designed.

The invention also encompasses said DNA primers.

Detection of a mutation in the *PRKAG3* gene may be performed by obtaining a sample of genomic DNA from a vertebrate, amplifying a segment of said DNA spanning a polymorphic marker by polymerase chain reaction using a set of primers of the invention, and detecting in said amplified DNA the presence of an allele of said polymorphic marker associated with said mutation.

By way of example, polymorphic markers which may be obtained according to the invention, and DNA primers allowing the detection of polymorphic markers closely linked to the *RN⁻* allele of porcine *PRKAG3* gene are listed in Table 1 hereinafter.

According to a preferred embodiment of the invention, the vertebrate is a mammal, preferably a farm animal and more preferably a porcine, and the mutation to be detected produces a functionally altered Prkag3. The detection of said mutation allows to predict whether said mammal or the progeny thereof is likely to have a high intramuscular glycogen concentration. An example of such a mutation produces a functionally altered Prkag3 having a R41Q substitution.

The present invention also includes kits for the practice of the methods of the invention. The kits comprise any container which contains at least one specific fragment of a nucleic acid sequence of the

invention, or at least one nucleic acid fragment able to specifically hybridise with a nucleic acid sequence of the invention. Said nucleic acid fragment may be labelled. The kits may also comprise a set of primers of the invention. They may be used in conjunction with commercially available amplification kits. They may also include positive or negative control reactions or markers, molecular weight size markers for gel electrophoresis, and the like.

Other kits of the invention may include antibodies of the invention, optionally labelled, as well as the appropriate reagents for detecting an antigen-antibody reaction. They may also include positive or negative control reactions or markers.

The invention further provides means for modulating the expression of vertebrate genes encoding a γ subunit of AMPK, and more specifically of the *PRKAG3* gene and/or the synthesis or activity of the products of said genes.

A purified AMPK heterotrimer comprising wild-type or mutant Prkag3 subunit, or a functionally altered mutant γ subunit having a mutation in the first CBS domain, may be used for screening *in vitro* compounds able to modulate AMPK activity, or to restore altered AMPK activity. This may be done, for instance, by:

- measuring the binding of the compound to said heterotrimer, using for example high-throughput screening methods; or,
- measuring changes in AMPK kinase activity, using for example high-throughput screening methods.

High throughput screening methods are disclosed, for instance, in "High throughput screening: The Discovery of Bioactive Substances", J.P. DEVLIN (Ed), MARCEL DEKKER Inc., New York (1997).

Nucleic acids of the invention may be used for therapeutic purposes. For instance, complementary

molecules or fragments thereof (antisense oligonucleotides) may be used to modulate AMPK activity, more specifically in muscular tissue.

Also, a nucleic acid sequence encoding a functional Prkag3 may be used for restoring a normal AMPK function.

Transformed cells or animal tissues expressing a wild-type or mutant Prkag3, or a functionally altered mutant of a γ subunit of AMPK as defined above, or expressing an AMPK comprising said mutant Prkag3, or said functionally altered mutant of a γ subunit of AMPK, may be used as *in vitro* model for elucidating the mechanism of AMPK activity or for screening compounds able to modulate the expression of AMPK.

The screening may be performed by adding the compound to be tested to the culture medium of said cells or said tissues, and measuring alterations in energy metabolism in said cells or said tissues using methods such as measurements of glucose concentrations (levels), glucose uptake, or changes of the ATP/AMP ratio, glycogen or lipid/protein content.

The invention provides animals transformed with a nucleic acid sequence of the invention.

In one embodiment, said animals are transgenic animals having at least a transgene comprising a nucleic acid of the invention.

In another embodiment, said animals are knockout animals. "Knockout animals" refers to animals whose native or endogenous *PRKAG3* alleles have been inactivated and which produce no functional Prkag3 of their own.

In light of the disclosure of the invention of DNA sequences encoding a wild-type or mutant Prkag3, or a functionally altered mutant of a γ subunit of AMPK, transgenic animals as well as knockout animals may be produced in accordance with techniques known in the art,

for instance by means of *in vivo* homologous recombination.

Suitable methods for the preparation of transgenic or knock-out animals are for instance disclosed in: *Manipulating the Mouse Embryo*, 2nd Ed., by HOGAN *et al.*, Cold Spring Harbor Laboratory Press, 1994; *Transgenic Animal Technology*, edited by C. PINKERT, Academic Press Inc., 1994; *Gene Targeting: A Practical Approach*, edited by A.L. JOYNER, Oxford University Press, 1995; *Strategies in Transgenic Animal Science*, edited y G.M. MONASTERSKY and J.M. ROBL, ASM Press, 1995; *Mouse Genetics: Concepts and Applications*, by Lee M. SILVER, Oxford University Press, 1995.

These animals may be used as models for metabolic diseases and disorders, more specifically for diseases and disorders of glycogen metabolism in muscle. For instance they may be used for screening test molecules. Transgenic animals may thus be used for screening compounds able to modulate AMPK activity. Knockout animals of the invention may be used, in particular, for screening compounds able to modulate energy metabolism, more specifically carbohydrate metabolism, in the absence of functional Prkag3.

The screening may be performed by administering the compound to be tested to the animal, and measuring alterations in energy metabolism in said animal using methods such as glucose tolerance tests, measurements of insulin levels in blood, changes of the ATP/AMP ratio, glycogen or lipid/protein content in tissues and cells.

Transgenic or knock-out farm animals with modified meat characteristics or modified energy metabolism may also be obtained.

The present invention will be further illustrated by the additional description which follows, which refers to examples of obtention and use of nucleic

acids of the invention. It should be understood however that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

EXAMPLE 1: ISOLATING THE *PRKAG3* GENE

We have screened a porcine Bacterial Artificial Chromosome (BAC) library (ROGEL-GAILLARD *et al.*, Cytogenet and Cell Genet, 851, 273-278, 1999) and constructed a contig of overlapping BAC clones across the region of pig chromosome 15 harbouring the *RN* gene. These BAC clones were in turn used to develop new genetic markers in the form of single nucleotide polymorphisms (SNPs) or microsatellites (MS) as described in Table 1 below.

Table 1

	Name of marker	BAC clone	Primer sequences	Size of PCR product (bp)	Marker type[a]	Alleles[b]
1	H3	115B9, 156E6, 361B4, 90A9	F; 5'-GGAATTTCAAGTCAGCCAAC-3' (SEQ ID NO: 5) R; 5'-CTTCAAAAGACCGTGCTACT-3' (SEQ ID NO: 6)	114 –138	MS	114, 126, 128, 132*, 134*, 136, 138
2	MS982H1	982H11	F; 5'-CTGGGAACCTCTATATGCTG-3' (SEQ ID NO: 7) R; 5'-TAGGGAAATACAAATCACAG-3' (SEQ ID NO: 8)	114 - 157	MS	114, 140, 142*, 144, 146, 150, 158
3	MS479L3	479L3, 297D7, 852B5, 153B5	F; 5'-CTCCAGCTCACAGGATGACA-3' (SEQ ID NO: 9) R; 5'-GTTTCTGCAGCTTTAGCATCTATTCC-3' (SEQ ID NO: 10)	150 - 164	MS	150*, 160, 162, 164
4	MS997M35	997F12	F; 5'-GAAGTATCCTGGGCTTCTGA-3' (SEQ ID NO: 11) R; 5'-GTTTCTCCAGGTTTCCAGACATCCAC-3' (SEQ ID NO: 12)	138 - 160	MS	138, 144, 152, 154, 160*
5	MS482H65	482E7	F; 5'-GCTTCTGTCTGCCCCTACTT-3' (SEQ ID NO: 13) R; 5'-GTTTCTAAGTTCTACTGTAAGACACC-3' (SEQ ID NO: 14)	78 - 90	MS	78, 80, 88*, 90
6	MS337H2	808G10, 947E5, 337G11	F; 5'-CCAAGCTGTGGTGGCTGAAT-3' (SEQ ID NO: 15) R; 5'-CAGCACAGCAGTGCCACCTA-3' (SEQ ID NO: 16)	145 - 165	MS	145, 149, 155, 161*, 165*
7	MS127B1	127G6, 134C9	F; 5'-CAAACTCTTCTAGGCGTGT-3' (SEQ ID NO: 17) R; 5'-GTTTCTGGAACTTCCATATGCCATGG-3' (SEQ ID NO: 18)	94 - 108	MS	94, 100, 108*, 114
8	CMKAR2	128A3, 337G11, 808G10, 947E7, 1110H12	F; 5'-AGGGTGGATGGTAGGCTTCA-3' (SEQ ID NO: 19) R; 5'-GTCTCGCTCCTGAAGGAAGT-3' (SEQ ID NO: 20)	208	SNP	112A*, 112T; 158A*, 158G 176A*, 176G
9	127G63	127G6, 134C9, 170D7, 1030A5,	F; 5'-AGTCACGTGGCCATGCTATC-3' (SEQ ID NO: 21) R; 5'-CTCAACTGGATTGAGTCAGT-3' (SEQ ID NO: 22)	409	SNP	234A*, 234C
10	VIL1	1088F2	F; 5'-TTGGCGCAACTGTTATTTCT-3' (SEQ ID NO: 23) R; 5'-AGGCAAAGGAAGAGCACAG-3' (SEQ ID NO: 24)	270	SNP	90T, 90G, 120A, 120G, 166C, 166T
11	NRAMP1	315F7, 530A6, 651C12, 1088F2, 1095H3	F; 5'-AGCCGTGGGCATCGTTGG-3' (SEQ ID NO: 25) R; 5'-AGAAGGAGACAGACAGGGCGA-3' (SEQ ID NO:26)	1300	RFLP (*StyI*)	1: 100+1200 bp 2:100+200+1000 bp

[a]MS=microsatellite; SNP=single nucleotide polymorphism.
[b]Microsatellite alleles are designated according to the length of the amplified fragment while SNPs are denoted according to the polymorphic nucleotide. Alleles associated with the *RN* allele are marked with an asterisk.

The new markers were used together with some previously described markers to construct a high-resolution linkage map. Standard linkage analysis using pedigree data comprising about 1,000 informative meioses for segregation at the *RN* locus made it possible to exclude *RN* from the region proximal to *MS479L3* and distal to microsatellite *Sw936*. Linkage Disequilibrium (LD) analysis was done with the same markers and a random sample of 68 breeding boars from the Swedish Hampshire population, scored for the RN phenotype by measuring glycogen content in muscle. The results of LD analysis using the DISMULT program (TERWILLIGER, Am. J. Hum. Genet., 56, 777-787, 1995) are shown in Figure 1. They reveal a sharp LD peak around the markers MS127B1 and SNP127G63. These markers appeared to show complete linkage disequilibrium with the *RN⁻* allele, i.e. *RN⁻* was associated with a single allele at these two loci. The most simple interpretation of this finding is that the *RN⁻* mutation arose on a chromosome carrying these alleles and that the two markers are so closely linked to the *RN* locus that the recombination frequency is close to 0%. The two markers are both present on the overlapping BAC clones 127G6 and 134C9 suggesting that the *RN* gene may reside on the same clone or one of the neighbouring clones.

A shot-gun library of the BAC clone 127G6 was constructed and more than 1,000 sequence reads were collected giving about 500,000 base pair random DNA sequence from the clone. The data were analysed and sequence contigs constructed with the PHRED, PHRAP and CONSED software package (University of Washington Genome Center, http://bozeman.mbt.washington.edu). The sequence data were masked for repeats using the REPEATMASKER software (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker) and BLAST searches were carried out using the NCBI web site (http://www.ncbi.nlm.nih.gov).

Three convincing matches to coding sequences were obtained. Two of these were against human cDNA sequences/genes, KIAA0173 described as being similar to pig tubulin-tyrosine ligase and located on HSA2q (UniGene cluster Hs.169910, http://www.ncbi.nlm.nih.gov/UniGene/) and CYP27A1 located on HSA2q33-ter (UniGene cluster Hs. 82568). The results strongly suggested that the pig coding sequences are orthologous to these human genes as it is well established that the *RN* region is homologous to HSA2q33-36 (ROBIC *et al.*, Mamm. Genome, 10, 565-568, 1999). However, none of these sequences appeared as plausible candidate genes for *RN*. The third coding sequence identified in BAC 127G6 showed highly significant sequence similarity to various AMP-activated protein kinase γ sequences including the yeast *SNF4* sequence. The cDNA sequence of this gene was determined by RT-PCR and RACE analysis using muscle mRNA from an rn^+/rn^+ homozygote. This sequence is shown in Figure 2 and in the enclosed sequence listing under SEQ ID NO: 1.

Legend of Figure 2:
5′ UTR: 5′ untranslated region
3′ UTR: 3′ untranslated region
CDS: coding sequence
***: stop codon
'-': identity to master sequence
'.': alignment gap

The frame of translation was determined on the basis of homology to other members in the protein family and assuming that the first methionine codon in frame is the start codon. The deduced polypeptidic sequence is shown in the enclosed sequence listing under SEQ ID NO: 2.

Figure 3 shows an amino acid alignment constructed with the CLUSTAL W program (THOMPSON *et al.*, Nucleic Acids Research, 22, 4673-4680, 1994) with

representative AMPK γ sequences in the nucleotide databases.

Legend of Figure 3:

Sequences used:

HumG1: Genbank U42412

MusG1: Genbank AF036535

HumG2: Human PRKAG2 (Genbank AJ249976)

PigG3: pig PRKAG3 (this study)

HumG3: human PRKAG3 (this study)

Dros: *Drosophila* (Genbank AF094764)

SNF4 (yeast): Genbank M30470

Both the PRKAG2 and *Drosophila* sequences have longer aminoterminal regions but they do not show significant homology to the aminoterminal region of PRKAG3 and were not included.

Abbreviations:

*: stop codon

'-': identity to master sequence

'.': alignment gap

The four CBS domains are overlined and the position of the *RN*⁻ mutation is indicated by an arrow.

Table 2 below shows the amino acid (above diagonal) and nucleotide sequence (below diagonal) identities (in %) among mammalian, *Drosophila* and yeast AMPKG/SNF4 sequences.

TABLE 2

	PigG3	HumG3	HumG1	RatG1	MusG1	HumG2	Dros	SNF4
PigG3	-	97.0	64.2	64.2	63.9	62.6	53.2	34.0
HumG3	90.7	-	63.6	63.6	63.6	62.6	53.5	34.4
HumG1	64.2	64.5	-	96.7	96.3	75.6	60.9	33.5
RatG1	65.8	65.8	88.0	-	97.4	75.3	61.1	33.5
MusG1	65.3	64.8	87.2	92.8	-	74.6	61.7	33.5
HumG2	61.6	61.6	68.1	67.8	65.9	-	63.1	34.5
Dros	58.4	58.4	59.0	59.3	59.0	60.0	-	36.2
SNF4	44.0	44.2	45.4	44.6	45.3	45.7	44.8	-

Figure 4 shows a Neighbor-Joining phylogenetic tree constructed with the PAUP software (SWOFFORD, *Phylogenetic analysis using parsimony (and other*

methods), Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, 1998) using yeast SNF4 as outgroup; support for branch orders obtained in bootstrap analysis with 1,000 replicates are indicated, scales of tree is indicated at the bottom. The result showed that the pig gene located in the *RN* region is distinct from mammalian *PRKAG1* and *PRKAG2* isoforms and most likely orthologous to a human gene represented by the human EST sequence AA178898 (GenBank) derived from a muscle cDNA library. This gene is herein denoted *PRKAG3* since it is the third isoform of a mammalian AMP-activated protein kinase γ characterised so far.

The cDNA sequence of this gene was determined by RT-PCR and 5'RACE analysis using human skeletal muscle cDNA (Clontech, Palo Alto, CA) and the deduced protein sequence showed 97% identity to the porcine sequence (Figure 2; Table 2). This cDNA sequence is also shown in the enclosed sequence listing under SEQ ID NO: 3; the deduced polypeptidic sequence is shown in the enclosed sequence listing under SEQ ID NO: 4.

Using the high resolution human TNG radiation hybrid panel (http://shgc-www.stanford.edu/RH/TNGindex.html), we mapped the human homologs of *PRKAG3*, *CYP27A1* and *KIAA0173*, all present in the porcine BAC127G6. The three genes are also very closely linked in the human genome. *PRKAG3* was mapped at a distance of 33 $cR_{50.000}$ from *KIAA0173* and 52 $cR_{50.000}$ from *CYP27A1*, with lod score support of 6.8 and 4.5, respectively.

The established role of AMPK in regulating energy metabolism, including glycogen storage, and its location in the region showing maximum linkage disequilibrium made *PRKAG3* a very strong candidate gene for *RN*. This was further strengthened by hybridisation analysis of a human multiple tissue northern blots (CLONTECH, Palo Alto, CA) using human *PRKAG1* (IMAGE clone

0362755 corresponding to GenBank entry AA018675), human *PRKAG2* (IMAGE clone 0322735 corresponding to GenBank entry W15439) and a porcine *PRKAG3* probe. The results are shown in Figure 5.

Legend of Figure 5:

H: Heart, B: Brain, Pl: Placenta, L: Lung, Li: Liver, M: Skeletal muscle, K: Kidney, Pa: Pancreas, S: Spleen, Th: Thymus, P: Prostate, T: Testis, O: Ovary, I: Small intestine, C: Colon (mucosal lining), PBL: Peripheral Blood Leukocyte.

While the *PRKAG1* and *PRKAG2* probes showed a broad tissue distribution of expression, *PRKAG3* showed a distinct muscle-specific expression. This result is also supported by the human EST database where multiple ESTs representing *PRKAG1* and *PRKAG2* have been identified in various cDNA libraries whereas a single EST (GenBank entry AA178898) representing *PRKAG3* has been obtained from a muscle cDNA library. The muscle-specific expression of *PRKAG3* and the lack of expression in liver are entirely consistent with the phenotypic effect of *RN⁻*, namely that glycogen content is altered in muscle but normal in liver (ESTRADE *et al.*, Comp. Biochem. Physiol. 104B, 321-326, 1993).

PRKAG3 sequences were determined from *rn⁺/rn⁺* and *RN⁻/RN⁻* homozygotes by RT-PCR analysis. A comparison revealed a total of seven nucleotide differences four of which were nonsynonymous substitutions was found between the sequence from rn^+ and RN^- animals, as shown in Table 3 below. Screening of these seven SNPs with genomic DNA from additional rn^+ and RN^- pigs of different breeds revealed five different *PRKAG3* alleles, but only the R41Q missense substitution was exclusively associated with *RN⁻*. This nonconservative substitution occurs in CBS1 which is the most conserved region among isotypic forms of the AMPK γ chain and arginine at this residue (number 70 in Prkag1) is conserved among different isoforms of

mammalian AMPK γ sequences as well as in the corresponding *Drosophila* sequence (Figure 3). A simple diagnostic DNA test for the R41Q mutation was designed based on the oligonucleotide ligation assay (OLA; LANDEGREN *et al.*, Science, 241, 1077-1080, 1988). Screening a large number of RN^- and rn^+ animals from the Hampshire breed as well as large number of rn+ animals from other breeds showed that the 41Q allele was present in all RN^- animals but not found in any rn^+ animals, as shown in Table 4 below. The absence of the 41Q allele from other breeds is consistent with the assumption that the *RN*$^-$ allele originated in the Hampshire breed; the allele has not yet been found in purebred animals from other breeds. In conclusion, the results provide convincing evidence that *PRKAG3* is identical to the *RN* gene and that the R41Q substitution most likely is the causative mutation.

Table 3. Comparison of the *PRKAG3* sequences associated with the *rn*$^+$ and *RN* alleles in different pig populations[a]

Allele	Associated *RN* allele	nt83	nt152	Codon 34	Codon 35	Codon 40	Codon 41	Codon 213	Population[b]
1	*RN*	ACC	CTC	GCC	CTG	GTC	CAA	TCT	H
		T	L	A	L	V	Q	S	
2	*rn*$^+$	---	---	---	---	---	-G-	---	L,LW,WB
		-	-	-	-	-	R	-	
3	*rn*$^+$	---	-C-	--T	T--	---	-G-	--C	H,L,LW,M,WB
		-	P	-	-	-	R	-	
4	*rn*$^+$	-A-	-C-	--T	T--	---	-G-	--C	D,H
		N	P	-	-	-	R	-	
5	*rn*$^+$	---	-C-	--T	T--	A--	-G-	--C	H,LW,WB
		-	P	-	-	I	R	-	

[a]nucleotide and codon numbers refer to the numbering of the sequence SEQ ID NO: 2

[b]H=Hampshire, L=Landrace, LW=Large White, M=Meishan, WB=Wild Boar, D=Duroc
N.D.=not determined, "-" indicates identity to the top sequence.

TABLE 4.

RN phenotype	Genotype at nucleotide 122			Total
	A/A	G/A	G/G	
RN^-, Hampshire[a]	40	87	0	127
RN^-, Hampshire[a,b]	0	13	0	13
m^+, Hampshire[a]	0	0	60	60
m^+, other breeds[c]	0	0	488	488

[a]represent both French and Swedish Hampshire populations
[b]heterozygosity *RN⁻/rn⁺* deduced using pedigree information
[c]breeds: Angler Saddleback, n=31; Blond Mangalitza, n=2; Bunte Bentheimer, n=16; Duroc, n=160; Göttinger Minipig, n=4; Landrace, n=83; Large White, n=72; Meishan, n=8; Piétrain, n=75; Red Mangalitza, n=5; Rotbunte Husumer, n=15; Schwalbenbauch Mangalitza, n=7; Schwäbisch Hällische, n=2; European Wild Boar, n=5; Japanese Wild Boar, n=3.

AMPK kinase activity was measured in muscle extracts from normal rn^+ pigs or from RN^- pigs in the presence or absence of AMP. Muscle tissue was collected from 4 rn+ (rn^+/rn^+) and 5 RN^- (2 RN^-/rn^+ - and 3 RN^-/RN^-) pigs. Muscle extract was purified up to and including the DEAE-Sepharose ion-exchange step as described by CARLING et al. [*Eur. J. Biochem.* 186, 129 (1989)]. AMPK activity was assayed by phosphorylation of the SAMS peptide [DAVIES et al., *Eur. J. Biochem.* 186, 123 (1989). Assays were performed in triplicates for each animal, both in the presence and absence of 200µM AMP. Since 0.1 µg of total protein from the 0.2M NaCl DEAE eluates showed a linear activity both in the presence and absence of added AMP for at least 12 minutes (data not shown) we used this amount of extract and an incubation time of 10 minutes in the experiments. For each assay, controls were included that lacked added peptide and the values presented represent peptide-dependent activity.

We found that AMPK kinase activity in muscle extracts was about three-fold higher in normal rn^+ pigs than in RN^- pigs both in the presence and absence of AMP (Table 4b)

Table 4b

RN type	*n*	Kinase activity[a] -AMP	+AMP
rn^+	4	955±134	1,519±323
RN^-	5	380±122	531±292
Significance		0.02	0.06

[a]Phosphorylation measured as cpm incorporated P^{32}/0.1µl muscle extract. Least square means ± standard errors are reported; the significance values were obtained by an F-test using a linear regression model.

Without being bound to any particular mechanism, it may be hypothesised that the AMPK heterotrimer including PRKAG3 is involved in the regulation of glucose transport into skeletal muscle.

It has recently been reported that AMPK activation induced by the AMP analogue AICAR or by muscle contraction leads to an increased glucose uptake in skeletal muscle (BERGERON *et al.*, Am. J. Physiol., 276, E938-944, 1999; HAYASHI *et al.*, Diabetes, 47, 1369-1373, 1998). If this is the function of the AMPK heterotrimer including PRKAG3, R41Q may be a gain-of-function mutation causing a constitutively active holoenzyme, for instance due to the loss of an inactivating allosteric site. If so, the reduced AMPK activity in RN^- animals is likely to reflect feed-back inhibition due to the high-energy status of the muscle. An increased uptake of glucose to skeletal muscle is expected to lead to an increase in muscle glycogen content as observed in RN^- animals. It has been shown that overexpression of glucose transporter 4 (GLUT4) in transgenic mice leads to increased uptake of glucose and increased glycogen storage (TREADWAY *et al.*, J. Biol. Chem., 269, 29956-29961, 1994). This type of gain-of-function model is consistent with the dominance of *RN*$^-$ as the presence of a single unregulated copy would have a large effect on AMPK enzyme activity.

An alternative hypothesis on the functional significance of the R41Q substitution associated with the *RN*⁻ allele may also be proposed. Based on the established roles of the yeast SNF1 enzyme in utilisation of glycogen and of mammalian AMPK for inhibiting energy-consuming pathways and stimulating energy-producing pathways, activated AMPK is expected to inhibit glycogen synthesis and stimulate glycogen degradation. If this is the functional role of the isoform(s) containing the *PRKAG3* product, the R41Q substitution would be a loss-of-function mutation or a dominant-negative mutation locking the AMPK heterotrimer in an inactive state, and thus inhibiting AMP activation and glycogen degradation. In these cases the phenotypic effect should be explained by haplo-insufficiency, since *RN*⁻ appears fully dominant.

R41Q may thus be a dominant negative mutation, but only if it interferes with multiple isoforms since the major AMPK activity in muscle appears to be associated with the PRKAG1 and 2 isoforms [CHEUNG, et al. *Biochem. J.* 346, 659 (2000)].

The distinct phenotype of the *RN*⁻ mutation indicates that PRKAG3 plays a key role in the regulation of energy metabolism in skeletal muscle. For instance, PRKAG3 is likely to be involved in the adaptation to physical exercise, which is associated with increased glycogen storage. It is also conceivable that loss-of-function mutations in *PRKAG3* (or other AMPK genes) may predispose individuals to noninsulin-dependent diabetes mellitus, and AMPK isoforms are potential drug targets for treatment of this disorder.

EXAMPLE 2: DETECTION OF THE R41Q SUBSTITUTION IN PIG *PRKAG3*

A part of *PRKAG3* including codon 41 was amplified in 10 µl reactions containing 100 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4.0 pmol of both forward (AMPKG3F3:5'-GGAGCAAATGTGCAGACAAG-3') and reverse

(AMPKG3R2:5'-CCCACGAAGCTCTGCTTCTT-3') primer, 10% DMSO, 1 U of *Taq* DNA polymerase and reaction buffer (ADVANCED BIOTECH, London, UK). The cycling conditions included an initial incubation at 94°C for 5 min followed by 3 cycles at 94°C (1 min), 57°C (1 min) and 72°C (1 min), and 35 cycles of 94°C (20 sec), 55°C (30 sec) and 72°C (30 sec). Allele discrimination at nucleotide position 122 was done using the oligonucleotide ligation assay (OLA, LANDEGREN *et al.*, Science, 241, 1077-1080, 1988). The OLA method was carried out as a gel-based assay. Each 10 µl OLA reaction contained 0.5 pmol of each probe SNPRN-A (5'Hex-TGGCCAACGGCGTCCA-3'), SNPRN-G (5'ROX-GGCCAACGGCGTCCG-3') and SNPRN-Common (5'phosphate-AGCGGCACCTTTGTGAAAAAAAAAA-3'), 1.5 U of thermostable AMPLIGASE and reaction buffer (EPICENTRE TECHNOLOGIES, Madison, WI) and 0.5 µl of the AMPKG3F3/AMPKG3R2 PCR product. After an initial incubation at 95°C for 5 min, the following thermocycling profile was repeated 10 times: denaturation at 94°C (30 sec), and probe annealing and ligation at 55°C (90 sec). After OLA cycling, 1 µl of product was heat denatured at 94°C (3 min), cooled on ice, and loaded onto 6% polyacrylamide denaturing gel for electrophoresis on an ABI377 DNA sequencer (PERKIN ELMER, Foster City, USA). The resulting fragment lengths and peak fluorescence were analysed using GENESCAN software (PERKIN ELMER, Foster City, USA).

The OLA-based method for the R41Q mutation was used to determine the genotype of DNA samples collected from 68 Swedish Hampshire animals phenotyped as either RN^- or rn^+ based on their glycolytic potential (GP) value. Figure 6 illustrates typical OLA results from the three possible genotypes. All RN^- animals were scored as homozygous A/A (n=28) or heterozygous A/G (n=36) at nucleotide position 122 whereas the rn^+ animals were homozygous G/G (n=4) at this position.

EXAMPLE 3: PREDICTING THE PRESENCE OF THE *RN*⁻ ALLELE USING A CLOSELY LINKED MICROSATELLITE, MS127B1

A microsatellite 127B1 (*MS127B1*) was cloned from BAC 127G7 containing pig *PRKAG3*. The BAC clone was digested with *Sau*3AI and the restriction fragments subcloned into the *Bam*HI site of pUC18. The resulting library was probed with a $(CA)_{15}$ oligonucleotide probe labelled with [γ-32P]-dATP. Strongly hybridising clones were sequenced and primers for PCR amplification of microsatellite loci were designed. Ten µl PCR reactions were performed containing 100 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4.0 pmol of both forward (MS127B1F:5′-Fluorescein-CAAACTCTTCTAGGCGTGT-3′) and reverse (MS127B1R:5′-GTTTCTGGAACTTCCATATGCCATGG-3′) primers, and 1 U of *Taq* DNA polymerase and reaction buffer (ADVANCED BIOTECH, London, UK). The cycling conditions included an initial incubation at 94°C for 5 min followed by 3 cycles at 94°C (1 min), 57°C (1 min) and 72°C (1 min), and 35 cycles of 94°C (20 sec), 55°C (30 sec) and 72°C (30 sec). The PCR products (0.3 µl) were separated using 4% polyacrylamide denaturing gel electrophoresis on an ABI377 DNA sequencer (PERKIN ELMER, Foster City, USA). The resulting fragment lengths were analysed using the GENESCAN and GENOTYPER software (PERKIN ELMER, Foster City, USA).

The method was used to determine the genotype of DNA samples collected from 87 Swedish Hampshire animals phenotyped as either RN^- or rn^+ based on their glycolytic potential (GP) value. Allele 108 (bp) showed a complete association to the *RN*⁻ allele in this material as all RN^- (*RN*⁻/ *RN*⁻ or *RN*⁻/*rn*⁺) animals were homozygous or heterozygous for this allele while no rn^+ (*rn*⁺/*rn*⁺) animals carried this allele, as shown in Table 5 below.

Table 5

Animals	n	Genotype				
		94/94	94/108	94/114	100/108	108/108
RN^-	80	0	37	0	2	41
rn^+	7	3	0	4	0	0

EXAMPLE 4: DETECTING THE PRESENCE OF THE *RN*⁻ ALLELE USING A PCR-RFLP TEST

The *RN*⁻ mutation inactivates a *Bsr*BI site GAG^CGG/CTC^GCC (*Bsr*BI RE site is not palindromic). At that site, the *RN*⁻ sequence is AAGCGG instead of GAGCGG.

A 134 bp long fragment of the *RN* gene is amplified from porcine genomic DNA. The rn^+ allele is identified after *Bsr*BI digestion, by detection of two fragments of 83 and 51 bps.

The test is performed as follows:

1° Primer sequences:

Sequence of primers used to amplify the *RN* mutation region:

RNU: 5′ GGGAACGATTCACCCTCAAC 3′

RNL: 5′ AGCCCCTCCTCACCCACGAA 3′

To provide an internal control of digestion, a *Bsr*BI site has been added at the extremity of one of the two primers within a 20 bp long tail. The tail permits both creation of a *Bsr*BI site (a shorter tail might be sufficient), and an easy discrimination of uncut fragment from other fragments. The use of tailed primers does not affect efficiency and specificity of amplification.

The sequence of the RNL modified primer including a control tail with a *Bsr*BI site is:

RNLBsrA14: 5′

$A_5C_2A_7$CCGCTCAGCCCCTCCTCACCCACGAA 3′

2° PCR reaction mixture used:

50 ng DNA

0.5 Unit *Taq* polymerase (GIBCO BRL)

1.5 mM $MgCl^2$

200 mM dNTP
0.2 µM each primer
Total reaction volume: 25 µl

3° PCR conditions used (on OMNIGENE HYBAID thermocycler):

1x (5min 95°C)
35x (45sec 57°C, 45sec72°C, 45sec95°C)
1x (45sec57°C, 15min 72°C)

4° Restriction enzyme digestion performed at 37°C for 2 hours:

10 µl PCR product
1x *Bsr*BI BIOLABS buffer
5U *Bsr*BI restriction enzyme (BIOLABS)
Total reaction volume: 15 µl

5° Size of fragments produced after PCR using primers with control tail and digestion with *Bsr*BI:

Uncut fragment from *RN*$^-$ or *rn*$^+$ allele : 154 bp
After digestion of fragment amplified from *RN*$^-$ allele : 137 bp + 17 bp
After digestion of fragment amplified from *rn*$^+$ allele : 83 bp + 54 bp + 17 bp

Size difference can be identified either after polyacrylamide, agarose/nusieve or agarose gel electrophoresis.

CLAIMS

1) A gamma subunit of a vertebrate AMP-activated kinase (AMPK), wherein said gamma subunit is a polypeptide having at least 70% identity with the polypeptide SEQ ID NO: 2.

2) A polypeptide of claim 1, wherein said polypeptide has at least 95% identity with the polypeptide SEQ ID NO:2.

3) A polypeptide of any of claims 1 or 2, wherein said polypeptide has the sequence SEQ ID NO: 2 or SEQ ID NO:4 or a sequence resulting from a V40I substitution in SEQ ID NO: 2.

4) A polypeptide which is a functionally altered mutant of a gamma subunit of a vertebrate AMP-activated kinase, wherein said polypeptide has at least a mutation located within the first CBS domain of said gamma subunit.

5) A polypeptide of claim 4, wherein the mutation is located within the region of the first CBS domain aligned with the region of a polypeptide of SEQ ID NO: 2 spanning from residue 30 to residue 50.

6) A polypeptide of claim 5, wherein the mutation is a R→Q substitution.

7) A polypeptide of claim 6, having a sequence resulting from a R41Q substitution in SEQ ID NO: 2.

8) A polypeptide which is a mutant of a gamma subunit of a vertebrate AMP-activated kinase, wherein said polypeptide results from a deletion of a part of a polypeptide of claim 1.

9) A nucleic acid sequence encoding a polypeptide of any of claims 1 to 8, or the complement thereof, provided that said nucleic acid sequence does not consist of the EST GENBANK AA178898.

10) A nucleic acid sequence of claim 9, having the sequence SEQ ID NO: 1 or SEQ ID NO:3, or the complement thereof.

11) A nucleic acid sequence comprising at least a portion of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8, and up to 500 kb of a 3′ and/or of a 5′ adjacent genomic DNA sequence, or the complement thereof.

12) A nucleic acid fragment selected among:

- a specific fragment of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8, or of a nucleic acid sequence of claim 11;
- a nucleic acid fragment which specifically hybridises under stringent conditions with a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8, or of a nucleic acid sequence of claim 11;

provided that said nucleic acid fragment does not consist of the EST GENBANK AA178898.

13) A set of primers for amplifying a nucleic acid sequence of any of claims 9 to 11 or a portion thereof, comprising at least a primer consisting of a nucleic acid fragment of claim 12.

14) A recombinant vector comprising a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8.

15) An host cell transformed by a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8.

16) A transgenic animal transformed by a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8.

17) A knockout animal, wherein the gene encoding a polypeptide of claim 1 is inactive.

18) A heterotrimeric AMPK wherein the γ subunit consists of a polypeptide of any of claims 1 to 8.

19) A method of detecting a metabolic disorder resulting from a mutation in a gene encoding a γ subunit of AMPK, wherein said process comprises:

- obtaining a nucleic acid sample from a vertebrate;

- checking the presence in said nucleic acid of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8, wherein said polypeptide is functionally altered.

20) A method of claim 19 wherein the disorder is correlated with an altered glycogen accumulation in the muscular cells and results from the expression of a functionally altered allele of a polypeptide of claim 1.

21) A method of any of claims 19 or 20 wherein the presence of the nucleic acid sequence encoding said mutant polypeptide is checked by contacting said nucleic acid sample with a nucleic acid probe obtained from a nucleic acid of claim 12 and spanning said mutation, under conditions of specific hybridisation between said probe and the mutant sequence to be detected, and detecting the hybridisation complex.

22) A method for obtaining a pair of primers allowing to detect a genetic polymorphic marker linked to a nucleic acid sequence encoding a polypeptide of claim 1, wherein said process comprises:

- screening a genomic DNA library from a vertebrate with a probe specific for a nucleic acid sequence encoding a polypeptide of claim 1, in order to select clones comprising said nucleic acid sequence and flanking chromosomal sequences;

- identifying a polymorphic locus in said flanking chromosomal sequences, and sequencing a DNA segment comprising said polymorphic locus ;

- designing primer pairs flanking said polymorphic locus.

23) A method of claim 22 wherein the selected clones comprise at least a portion of a nucleic acid sequence encoding a polypeptide of claim 1, and up to 500 kb of a 3′ and/or of a 5′ adjacent sequence.

24) A method of any of claims 19 to 23 wherein the vertebrate is a mammal.

25) A method of claim 24 wherein said mammal is a pig.

26) A pair of primers obtainable by the process of any of claims 21 to 25.

27) A process for detecting a dysfunction of carbohydrate metabolism resulting from the expression of a functionally altered allele of a polypeptide of claim 1 in a vertebrate, wherein said process comprises:

- obtaining a sample of genomic DNA from said vertebrate;

- contacting said DNA with a pair of primers of claim 26 under conditions allowing PCR amplification;

- analysing the PCR product to detect if an allele of a polymorphic marker linked to a nucleic acid sequence encoding a functionally altered allele of a polypeptide of claim 1 is present.

28) A process of claim 27, wherein said functionally altered polypeptide results from a R41Q substitution.

29) A process of any of claims 27 or 28, wherein said vertebrate is a mammal.

30) A process of claim 29 wherein said mammal is a pig.

31) A process of claim 30 wherein the pair of primers is selected among:

- a pair of primers consisting of SEQ ID NO: 5 and SEQ ID NO: 6;

- a pair of primers consisting of SEQ ID NO: 7 and SEQ ID NO: 8;

- a pair of primers consisting of SEQ ID NO: 9 and SEQ ID NO: 10;

- a pair of primers consisting of SEQ ID NO: 11 and SEQ ID NO: 12;

- a pair of primers consisting of SEQ ID NO: 13 and SEQ ID NO: 14;
- a pair of primers consisting of SEQ ID NO: 15 and SEQ ID NO: 16;
- a pair of primers consisting of SEQ ID NO: 17 and SEQ ID NO: 18;
- a pair of primers consisting of SEQ ID NO: 19 and SEQ ID NO: 20;
- a pair of primers consisting of SEQ ID NO: 21 and SEQ ID NO: 22;
- a pair of primers consisting of SEQ ID NO: 23 and SEQ ID NO: 24;
- a pair of primers consisting of SEQ ID NO: 25 and SEQ ID NO: 26.

32) Use of a transformed cell of claim 15 to screen compounds able to modulate AMPK activity.

33) Use of a transgenic animal of claim 16 to screen compounds able to modulate AMPK activity.

34) Use of a knockout animal of claim 17 to screen compounds able to modulate energy metabolism in the absence of a functional polypeptide of claim 1.

35) Use of an heterotrimeric AMPK of claim 18 to screen compounds able to modulate AMPK activity.

ABSTRACT

The invention concerns variants of the gamma chain of vertebrate AMP-activated kinase (AMPK), as well as nucleic acid sequences encoding said variants and use thereof for the diagnosis or treatment of dysfunction of energy metabolisms.

Figure 1. Linkage disequilibrium in the *RN* region

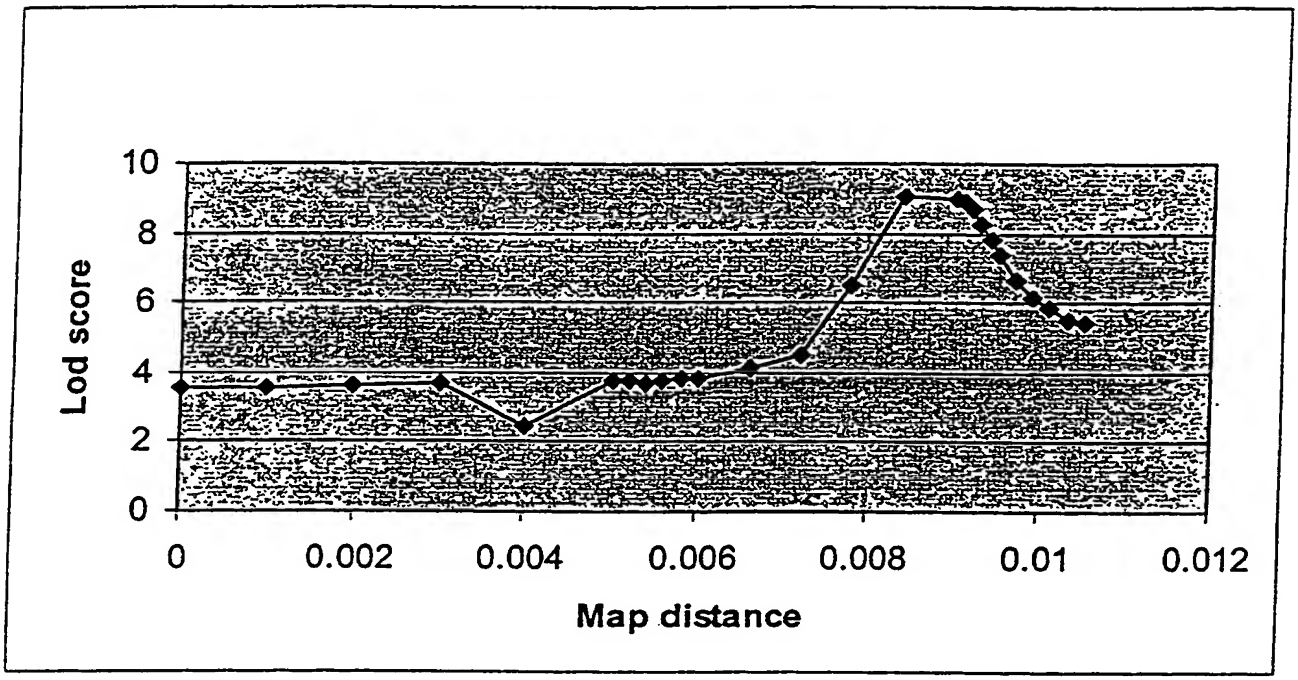


Figure 2. Alignment of pig and human *PRKAG3* cDNA sequences and deduced amino acid sequences

```
          5'UTR   10        20        30        40        50        60        70        80
Pig       TTCCTAGAGCAAGGAGAGAGCCGTTCATGGCCATCCCGAGCTGTAACCACCAGCTCAGAAAGAAGCCATGGGGACCAGGG
Hum       ----------A-A-C---A-C----------A-C------G----G----------T--G----A-A-G-A-
                90        100       110       120       130       140       150       160
Pig       GAACAAGGCCTCTAGATGGACAAGGCAGGAGGATGTAGAGGAAGGGGGGCCTCCGGGCCCGAGGGAAGGTCCCCAGTCCA
Hum       -GC---A----TG--------------A--TCG--G----------A---A--A--T-A-G----------G-----
                170       180       190       200       210       220       230       240
Pig       GGCCAGTTGCTGAGTCCACCGGGCAGGAGGCCACATTCCCCAAGGCCACACCCTTGGCCCAAGCCGCTCCCTTGGCCGAG
Hum       -----AC----------------T----------------A----------T-----T-A---...T----G-
                250       260       270       280       290       300       310       320
Pig       GTGGACAACCCCCCAACAGAGCGGGACATCCTCCCCTCTGACTGTGCAGCCTCAGCCTCCGACTCCAACACAGACCATCT
Hum       ----G--CT--A-------G-T-----TG----------A----------TG-A-G------G-----TG--G-
                330       340       350       360       370       380       390       400
Pig       GGATCTGGGCATAGAGTTCTCAGCCTCGGCGGCGTCGGGGGATGAGCTTG...GGCTGGTGGAAGAGAAGCCAGCCCCGT
Hum       ---G----C--CG------C-----A-A-A---C-G--A-TG------A-AAG-C---C---------G---T----T--
                410       420       430       440       450       460       470 5'UTR
Pig       GCCCATCCCCAGAGGTGCTGTTACCCAGGCTGGGCTGGGATGATGAGCTGCAGAAGCCGGGGGCCCAGGTCTAC
Hum       ---TG-----GC---CC-CA--T----A----------C--A----G---A--C--C------A-----
          CDS
                                              10                                  20
Pig       ATG CAC TTC ATG CAG GAG CAC ACC TGC TAC GAT GCC ATG GCG ACC AGC TCC AAA CTG GTC
          Met His Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr Ser Ser Lys Leu Val
Hum       --- -G- --- --- --- --- --- --- --- --- --- --- --- --A --T --- --- --G --A ---
           -  Arg  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                              30                                  40
Pig       ATC TTC GAC ACC ATG CTG GAG ATC AAG AAG GCC TTC TTT GCC CTG GTG GCC AAC GGC GTC
          Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe Phe Ala Leu Val Ala Asn Gly Val
Hum       --- --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --T --G
           -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                              50                                  60
Pig       CGA GCG GCA CCT TTG TGG GAC AGC AAG AAG CAG AGC TTC GTG GGG ATG CTG ACC ATC ACA
          Arg Ala Ala Pro Leu Trp Asp Ser Lys Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr
Hum       --G --A --C --- C-A --- --- --- --- --- --- --- --T --- --- --- --- --- --- --T
           -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                              70                                  80
Pig       GAC TTC ATC TTG GTG CTG CAC CGC TAT TAC AGG TCC CCC CTG GTC CAG ATC TAC GAG ATT
          Asp Phe Ile Leu Val Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile
Hum       --- --- --- C-- --- --- --T --- --C --- --- --- --- --- --- --- --- --T --- ---
           -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                              90                                 100
Pig       GAA GAA CAT AAG ATT GAG ACC TGG AGG GAG ATC TAC CTT CAA GGC TGC TTC AAG CCT CTG
          Glu Glu His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe Lys Pro Leu
Hum       --- C-- --- --- --- --- --- --- --- --- --- --- --G --- --- --- --- --- --- ---
           -  Gln  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                             110                                 120
Pig       GTC TCC ATC TCT CCC AAT GAC AGC CTG TTC GAA GCT GTC TAC GCC CTC ATC AAG AAC CGG
          Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val Tyr Ala Leu Ile Lys Asn Arg
Hum       --- --- --- --- --T --- --T --- --- --T --- --- --- --- A-- --- --- --- --- ---
           -   -   -   -   -   -   -   -   -   -   -   -   -   -  Thr  -   -   -   -   -
                                             130                                 140
Pig       ATC CAC CGC CTG CCG GTC CTG GAC CCT GTC TCC GGG GCT GTG CTC CAC ATC CTC ACA CAT
          Ile His Arg Leu Pro Val Leu Asp Pro Val Ser Gly Ala Val Leu His Ile Leu Thr His
Hum       --- --T --- --- --T --T --T --- --G --G --A --C AAC --A --- --- --- --- --- --C
           -   -   -   -   -   -   -   -   -   -   -   -  Asn  -   -   -   -   -   -   -
                                             150                                 160
Pig       AAG CGG CTT CTC AAG TTC CTG CAC ATC TTT GGC ACC CTG CTG CCC CGG CCC TCC TTC CTC
          Lys Arg Leu Leu Lys Phe Leu His Ile Phe Gly Thr Leu Leu Pro Arg Pro Ser Phe Leu
Hum       --A --C --G --- --- --- --- --- --- --- --- --T T-- --- --- --- --- --- --- ---
           -   -   -   -   -   -   -   -   -   -   -   - Ser  -   -   -   -   -   -   -
```

```
                                                  170                                                 180
Pig   TAC CGC ACC ATC CAA GAT TTG GGC ATC GGC ACA TTC CGA GAC TTG GCC GTG GTG CTG GAA
      Tyr Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala Val Val Leu Glu
Hum   --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --G
       -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

                                                  190                                                 200
Pig   ACG GCG CCC ATC CTG ACC GCA CTG GAC ATC TTC GTG GAC CGG CGT GTG TCT GCG CTG CCT
      Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val Asp Arg Arg Val Ser Ala Leu Pro
Hum   --A --A --- --- --- --T --- --- --- --- --T --- --- --- --- --- --- --A --- ---
       -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

                                                  210                                                 220
Pig   GTG GTC AAC GAA ACT GGA CAG GTA GTG GGC CTC TAC TCT CGC TTT GAT GTG ATC CAC CTG
      Val Val Asn Glu Thr Gly Gln Val Val Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu
Hum   --- --- --- --- TG- --T --- --C --- --- --- --T --C --- --- --- --- --T --- ---
       -   -   -   -  Cys  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

                                                  230                                                 240
Pig   GCT GCC CAA CAA ACA TAC AAC CAC CTG GAC ATG AAT GTG GGA GAA GCC CTG AGG CAG CGG
      Ala Ala Gln Gln Thr Tyr Asn His Leu Asp Met Asn Val Gly Glu Ala Leu Arg Gln Arg
Hum   --- --- --G --- --C --- --- --- --- --- --- -G- --- --- --- --- --- --- --- A--
       -   -   -   -   -   -   -   -   -   -   -  Ser  -   -   -   -   -   -   -   -

                                                  250                                                 260
Pig   ACA CTG TGT CTG GAA GGC GTC CTT TCC TGC CAG CCC CAC GAG ACC TTG GGG GAA GTC ATT
      Thr Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Thr Leu Gly Glu Val Ile
Hum   --- --A --- --- --G --A --- --- --- --- --- --- --- --- -G- --- --- --- --G --C
       -   -   -   -   -   -   -   -   -   -   -   -   -   -  Ser  -   -   -   -   -

                                                  250                                                 260
Pig   GAC CGG ATT GTC CGG GAA CAG GTG CAC CGC CTG GTG CTC GTG GAT GAG ACC CAG CAC CTT
      Asp Arg Ile Val Arg Glu Gln Val His Arg Leu Val Leu Val Asp Glu Thr Gln His Leu
Hum   --- A-- --- -CT --- --G --- --A --- A-G --- --- --A --- --C --- --- --- --T --C
       -   -   -  Ala  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

                                                  270                                                 280
Pig   CTG GGC GTG GTG TCC CTC TCT GAC ATC CTT CAG GCT CTG GTG CTC AGC CCT GCT GGA ATT
      Leu Gly Val Val Ser Leu Ser Asp Ile Leu Gln Ala Leu Val Leu Ser Pro Ala Gly Ile
Hum   T-- --- --- --C --- --- --C --- --- --- --- --A --- --- --- --- --- --- --C --C
       -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

                        CDS
Pig   GAT GCC CTC GGG GCC TGA
      Asp Ala Leu Gly Ala ***
Hum   --- --- --- --- --- ---
       -   -   -   -   -   -

      3'UTR   10          20          30          40          50          60          70          80
Pig   GAACCTTGGAACCTTTGCTCTCAGGCCACCTGGCACACCTGGAAGCCAGTGAAGGGAGCCGTGGACTCAGCTCTCACTTC
Hum   ---GA-CT--GT-C-CAA--C--A----A---.-----.---------A------A-T...--AGAA-----.---T---
             90          100         110         120         130         140         150         160
Pig   CCCTCAGCCCCACTTGCTGGTCTGGCTCTTGTTCAGGTAGGCTCCGCCCGGGGC.....CCCTGGCCTCAGCATCAGCCC
Hum   ---A.-C---A-T--------TCA---A-GA------CTTCT--A---TTCCAAAATTG--T-T----T-.-T-GT--T-
            170         180         190         200         210         220         230         240
Pig   CTCAGTCTCCCT.GGGCACCCAGATCTCAGACTGGGGCACCCTGAAGATG.GGAGTGGCCCAGCTTATAGCTGAGCAG.C
Hum   -.---AAC--T-C-----TG-CC-GTG--CCA----..-TGA----AT-AA----AACAG-T-AG-CA----TG-AG-T-
            250         260         270         280         290         300         310         320
Pig   CTTGTG..AAATCTACCAGCATCAAGACT...CACTGTGGGACCACTGCTTTG...TCCCATTCTCAGCTGAAATGAT.G
Hum   -C--AACC-G-GGC--T--G--T-CCC-AGGG-CA-C--T-CT-CA---CCGCCCA----C--GC-GC----CTG-G-C-
            330         340         350         360         370         380         390         400
Pig   GAGGGCCTCATAAGAGGGGTGGACAGGGC..CTGGAGTAGAGGCCAGATCAGTGACGT..GCCTTCAGG....ACCTCCG
Hum   --T----C--GTG-..-TT-A-T------TT-----T-CCTC-GTTTC-GG-CT--C-AT-G------CCTTC-G----T
            410         420         430         440         450         460         470         480
Pig   GGGAGTTAGAGCTGCCCTCTCTCAGTT.......CAGTTCCCCCCTGCTGAGAATG.TCCCTGGAAGGAAGCCAGTTAAT
Hum   ------CCC-----TTG-.--C---CAACGTCGC--C-G---T---A--CTCC-G-C-TTG-CATTTC---G-T-C-G-.
            490         500         510         520         530         540         550         560
Pig   AAACCTTGGTTGGATGGAATTTCCACACTCG.....................................................
Hum   --TG--GCA--TC-G--G....----CA-G-AGCAGCCGTTATTTATAGAACTGCCTGTTGGAGGTGGGGAGTCCTCCCT
            570         580         590         600         610         620         630         640
Pig   .....................................................................................
Hum   CCATTCTTGTCCAGAAAACTCCTTAGCTCTCGCAGTGAGCCATGTTCTTAGTCTCCAGGGATGGATGGCCTTGTATATGG
```

```
                650       660       670       680       690       700       710       720
Pig  ..................................................................................
Hum  ACCCCTGAGAATGAGCAATTGAGAAAACAAAACAAAAGGAACAATCCATGAACTTAGATTTTATTGGTTTCACTCAAAAT

                730       740
Pig  ..................
Hum  GCTGCAGTCATTTGACCTG
```

A

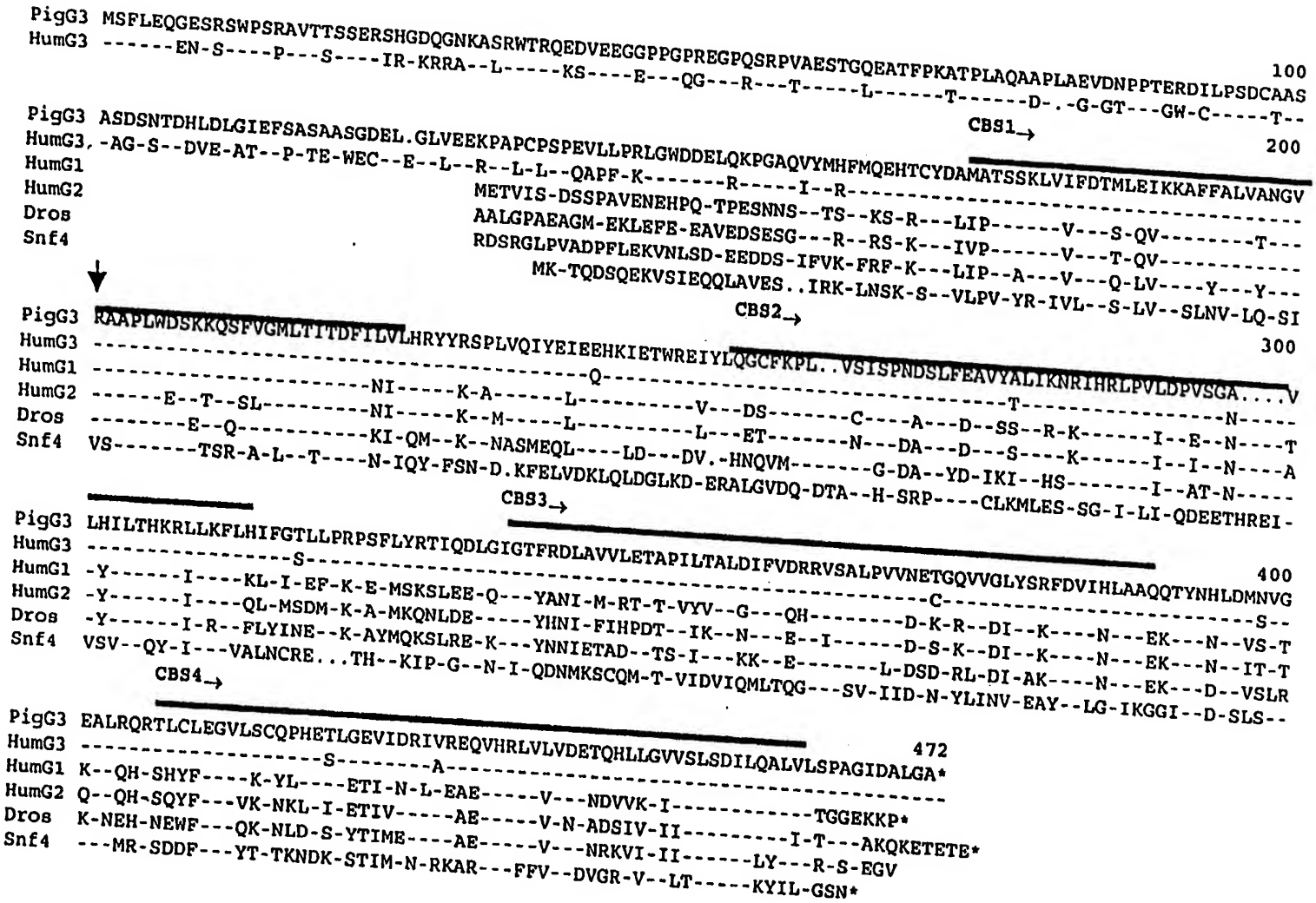


Figure 3

Figure 4. Neighbor-joining tree constructed for mammalian, *Drosophila* and yeast AMPKG/SNF4 amino acid sequences.

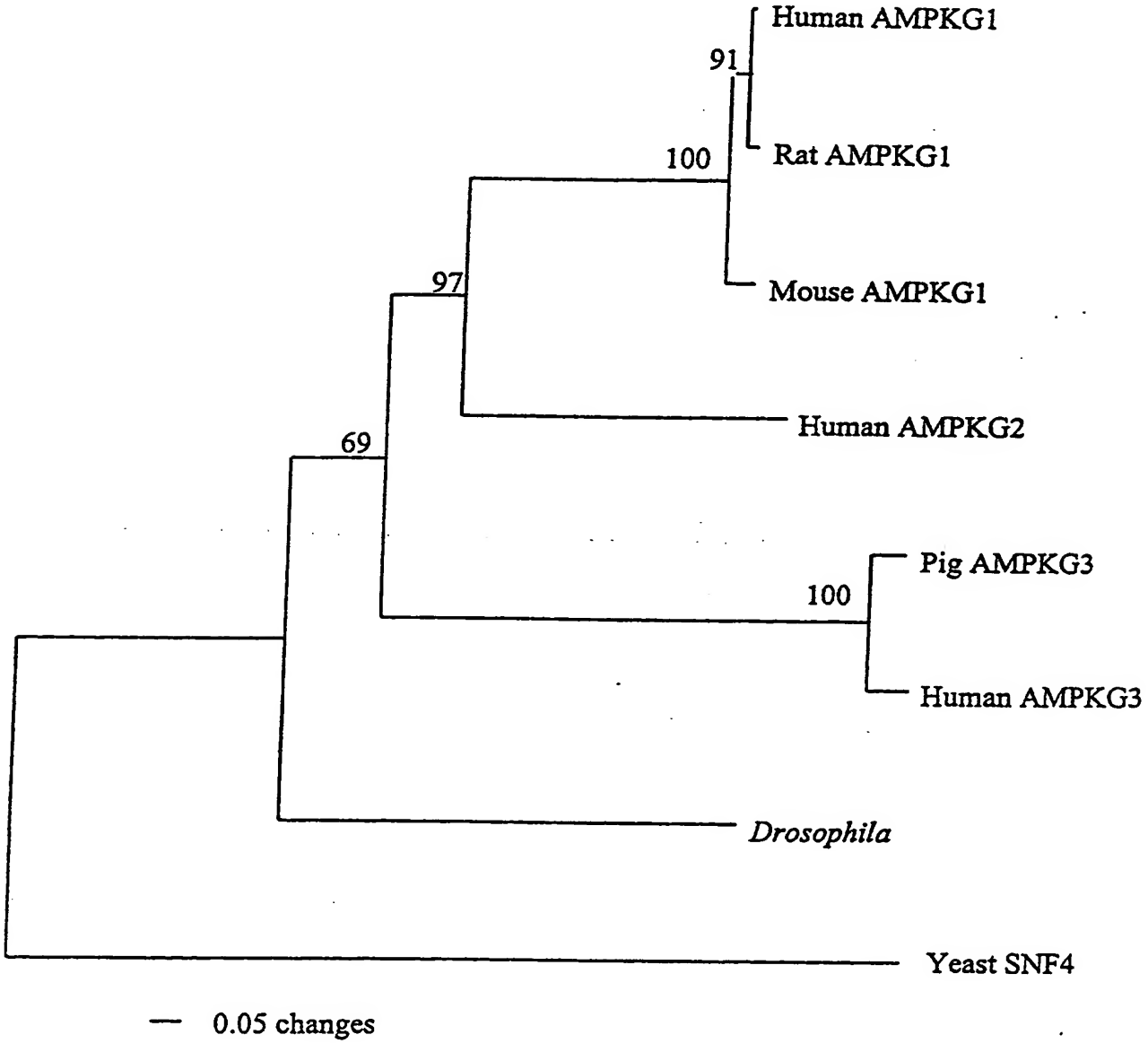


Figure 5. Northern blot analysis of human mRNA using human *PRKAG1*, human *PRKAG2* and porcine *PRKAG3* probes

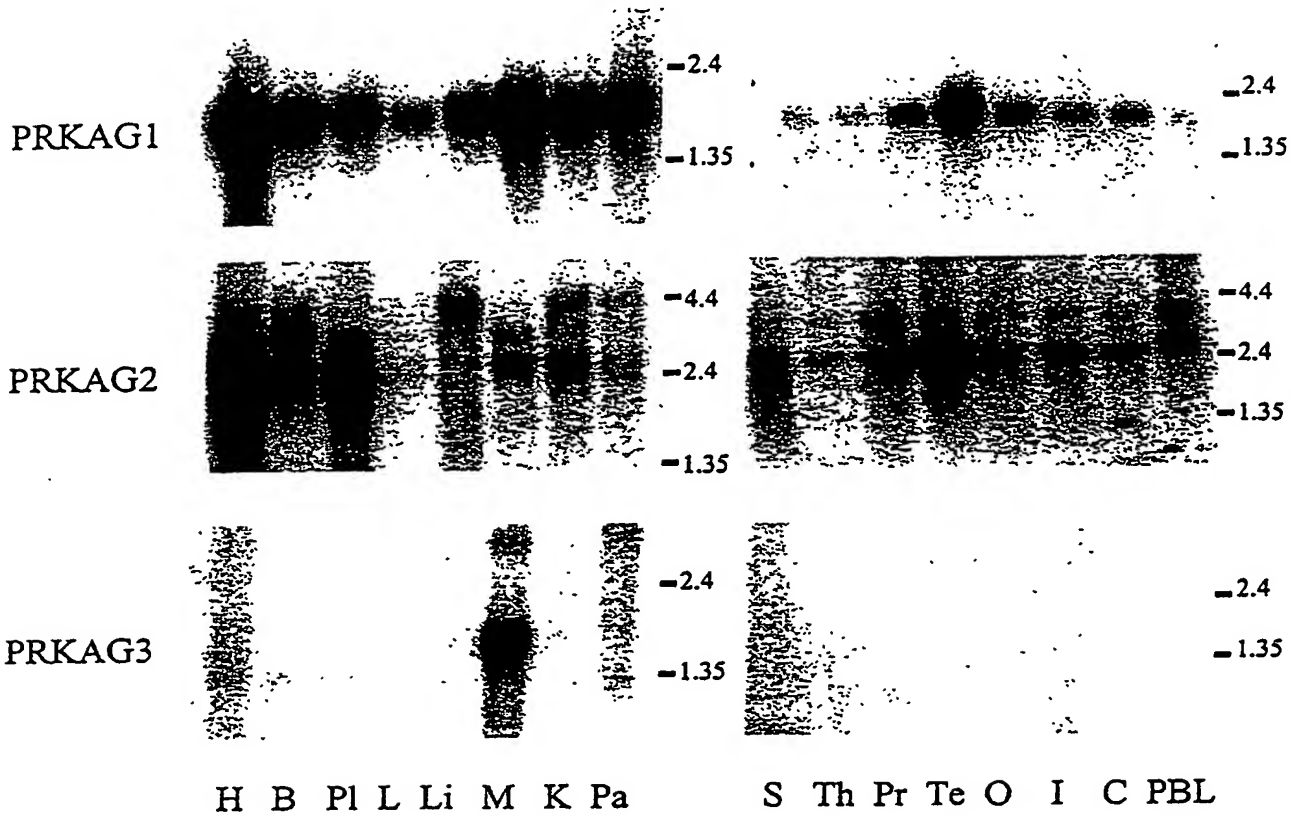
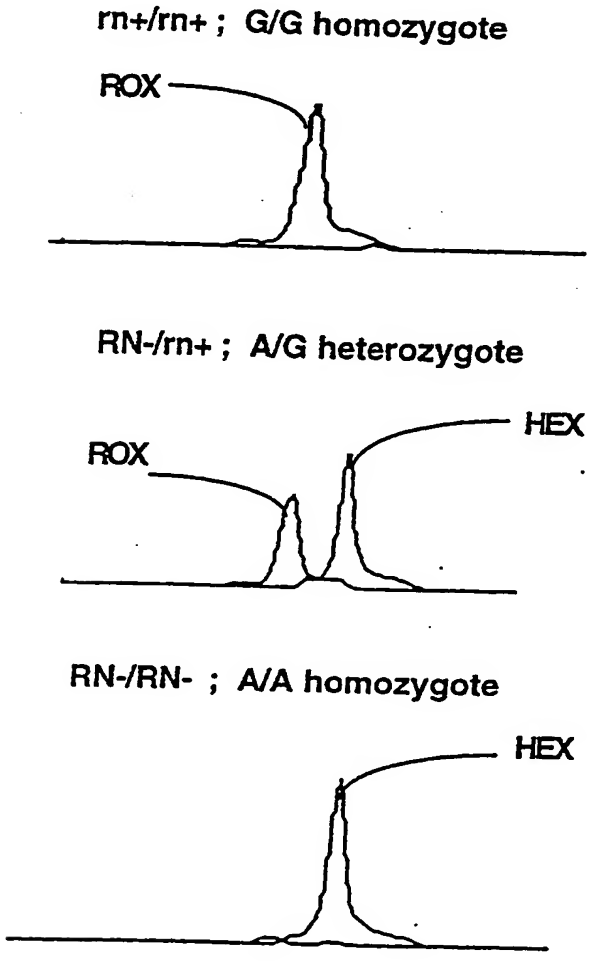


Figure 6. Detection of the missense mutation at nucleotide position 122 in *PRKAG3* associated with *RN-* allele in pigs using the OLA method.



Pig PRKAG3 Splice variant 1

ATG GAG CTT GCC GAG CTA GAG CAG GCA CTG CGC AGG GTC CCG GGG TCC CGG GGG
GGC TGG GAG CTG GAG CAA CTG AGG CCA GAG GGC AGA GGG CCC ACC ACT GCG GAT
ACT CCC TCC TGG AGC AGC CTC GGG GGA CCT AAG CAT CAA GAG ATG AGC TTC CTA
GAG CAA GGA GAG AGC CGT TCA TGG CCA TCC CGA GCT GTA ACC ACC AGC TCA GAA
AGA AGC CAT GGG GAC CAG GGG AAC AAG GCC TCT AGA TGG ACA AGG CAG GAG GAT
GTA GAG GAA GGG GGG CCT CCG GGC CCG AGG GAA GGT CCC CAG TCC AGG CCA GTT
GCT GAG TCC ACC GGG CAG GAG GCC ACA TTC CCC AAG GCC ACA CCC TTG GCC CAA
GCC GCT CCC TTG GCC GAG GTG GAC AAC CCC CCA ACA GAG CGG GAC ATC CTC CCC
TCT GAC TGT GCA GCC TCA GCC TCC GAC TCC AAC ACA GAC CAT CTG GAT CTG GGC
ATA GAG TTC TCA GCC TCG GCG GCG TCG GGG GAT GAG CTT GGG CTG GTG GAA GAG
AAG CCA GCC CCG TGC CCA TCC CCA GAG GTG CTG TTA CCC AGG CTG GGC TGG GAT
GAT GAG CTG CAG AAG CCG GGG GCC CAG GTC TAC ATG CAC TTC ATG CAG GAG CAC
ACC TGC TAC GAT GCC ATG GCG ACC AGC TCC AAA CTG GTC ATC TTC GAC ACC ATG
CTG GAG ATC AAG AAG GCC TTC TTT GCC CTG GTG GCC AAC GGC GTC CGA GCG GCA
CCT TTG TGG GAC AGC AAG AAG CAG AGC TTC GTG GGG ATG CTG ACC ATC ACA GAC
TTC ATC TTG GTG CTG CAC CGC TAT TAC AGG TCC CCC CTG GTC CAG ATC TAC GAG
ATT GAA GAA CAT AAG ATT GAG ACC TGG AGG GAG ATC TAC CTT CAA GGC TGC TTC
AAG CCT CTG GTC TCC ATC TCT CCC AAT GAC AGC CTG TTC GAA GCT GTC TAC GCC
CTC ATC AAG AAC CGG ATC CAC CGC CTG CCG GTC CTG GAC CCT GTC TCC GGG GCT
GTG CTC CAC ATC CTC ACA CAT AAG CGG CTT CTC AAG TTC CTG CAC ATC TTT GGC
ACC CTG CTG CCC CGG CCC TCC TTC CTC TAC CGC ACC ATC CAA GAT TTG GGC ATC
GGC ACA TTC CGA GAC TTG GCC GTG GTG CTG GAA ACG GCG CCC ATC CTG ACC GCA
CTG GAC ATC TTC GTG GAC CGG CGT GTG TCT GCG CTG CCT GTG GTC AAC GAA ACT

GGA CAG GTA GTG GGC CTC TAC TCT CGC TTT GAT GTG ATC CAC CTG GCT GCC CAA
CAA ACA TAC AAC CAC CTG GAC ATG AAT GTG GGA GAA GCC CTG AGG CAG CGG ACA
CTG TGT CTG GAA GGC GTC CTT TCC TGC CAG CCC CAC GAG ACC TTG GGG GAA GTC
ATT GAC CGG ATT GTC CGG GAA CAG GTG CAC CGC CTG GTG CTC GTG GAT GAG ACC

Figure 7a

CAG CAC CTT CTG GGC GTG GTG TCC CTC TCT GAC ATC CTT CAG GCT CTG GTG CTC
AGC CCT GCT GGA ATT GAT GCC CTC GGG GCC TGA GAA CCT TGG AAC CTT TGC TCT
CAG GCC ACC TGG CAC ACC TGG AAG CCA GTG AAG GGA GCC GTG GAC TCA GCT CTC
ACT TCC CCT CAG CCC CAC TTG CTG GTC TGG CTC TTG TTC AGG TAG GCT CCG CCC
GGG GCC CCT GGC CTC AGC ATC AGC CCC TCA GTC TCC CTG GGC ACC CAG ATC TCA
GAC TGG GGC ACC CTG AAG ATG GGA GTG GCC CAG CTT ATA GCT GAG CAG CCT TGT
GAA ATC TAC CAG CAT CAA GAC TCA CTG TGG GAC CAC TGC TTT GTC CCA TTC TCA
GCT GAA ATG ATG GAG GGC CTC ATA AGA GGG GTG GAC AGG GCC TGG AGT AGA GGC
CAG ATC AGT GAC GTG CCT TCA GGA CCT CCG GGG AGT TAG AGC TGC CCT CTC TCA
GTT CAG TTC CCC CCT GCT GAG AAT GTC CCT GGA AGG AAG CCA GTT AAT AAA CCT
TGG TTG GAT GGA ATT TGG AGA GTC

Figure 7a (continued)

Pig PRKAG3 splice variant 2

MELAELEQAL RRVPGSRGGW ELEQLRPEGR GPTTADTPSW SSLGGPKHQE MSFLEQGESR
SWPSRAVTTS SERSHGDQGN KASRWTRQED VEEGGPPGPR EGPQSRPVAE STGQEATFPK
ATPLAQAAPL AEVDNPPTER DILPSDCAAS ASDSNTDHLD LGIEFSASAA SGDELGLVEE
KPAPCPSPEV LLPRLGWDDE LQKPGAQVYM HFMQEHTCYD AMATSSKLVI FDTMLEIKKA
FFALVANGVR AAPLWDSKKQ SFVGMLTITD FILVLHRYYR SPLVQIYEIE EHKIETWREI
YLQGCFKPLV SISPNDSLFE AVYALIKNRI HRLPVLDPVS GAVLHILTHK RLLKFLHIFG
TLLPRPSFLY RTIQDLGIGT FRDLAVVLET APILTALDIF VDRRVSALPV VNETGQVVGL
YSRFDVIHLA AQQTYNHLDM NVGEALRQRT LCLEGVLSCQ PHETLGEVID RIVREQVHRL
VLVDETQHLL GVVSLSDILQ ALVLSPAGID ALGA*

Figure 7b

Pig PRKAG3 Splice variant 2

CT CAG AGG GGG ACA TCT GGA CAC ACA CTG GCC ATC CTC CCG GGA CCA CCC ACG AAC TCC
GCC TCC CCA GCC TCC CAG CTG GCT GCA ACT AGC CCT TTC TAA CCC CAG TGC CAC CCA CGA
CCG GAG TTT GTA CAA GGA AGA AAG TTA AGC TCA ACT TGC TTG CAT TTT CCC TCC TTT GGC
GGC TGG CAA ATG AGA GAG GCC CTC ACG GTG GGA ACA CAG CGG GGG CTC TGA GAG CAT ACC
CTC CTC CCA CCC CAG TTC CAA GCT GCT TCC CCC ACA GAG GAT CCT TCT CAC CTG TCC TCA
GAG ACC CAG GAG ACA GCC CGG GAC CAG GCA TCA AGA TTC CAG ACT CCC TCC TGG AGC AGC
CTC GGG GGA CCT AAG CAT CAA GAG ATG AGC TTC CTA GAG CAA GGA GAG AGC CGT TCA TGG
CCA TCC CGA GCT GTA ACC ACC AGC TCA GAA AGA AGC CAT GGG GAC CAG GGG AAC AAG GCC
TCT AGA TGG ACA AGG CAG GAG GAT GTA GAG GAA GGG GGG CCT CCG GGC CCG AGG GAA GGT
CCC CAG TCC AGG CCA GTT GCT GAG TCC ACC GGG CAG GAG GCC ACA TTC CCC AAG GCC ACA
CTC CCC TCT GAC TGT GCA GCC TCA GCC TCC GAC TCC AAC ACA GAC CAT CTG GAT CTG GGC
ATA GAG TTC TCA GCC TCG GCG GCG TCG GGG GAT GAG CTT GGG CTG GTG GAA GAG AAG CCA
GCC CCG TGC CCA TCC CCA GAG GTG CTG TTA CCC AGG CTG GGC TGG GAT GAT GAG CTG CAG
AAG CCG GGG GCC CAG GTC TAC ATG CAC TTC ATG CAG GAG CAC ACC TGC TAC GAT GCC ATG
GCG ACC AGC TCC AAA CTG GTC ATC TTC GAC ACC ATG CTG GAG ATC AAG AAG GCC TTC TTT
GCC CTG GTG GCC AAC GGC GTC CGA GCG GCA CCT TTG TGG GAC AGC AAG AAG CAG AGC TTC
GTG GGG ATG CTG ACC ATC ACA GAC TTC ATC TTG GTG CTG CAC CGC TAT TAC AGG TCC CCC
CTG GTC CAG ATC TAC GAG ATT GAA GAA CAT AAG ATT GAG ACC TGG AGG GAG ATC TAC CTT
CAA GGC TGC TTC AAG CCT CTG GTC TCC ATC TCT CCC AAT GAC AGC CTG TTC GAA GCT GTC
TAC GCC CTC ATC AAG AAC CGG ATC CAC CGC CTG CCG GTC CTG GAC CCT GTC TCC GGG GCT
GTG CTC CAC ATC CTC ACA CAT AAG CGG CTT CTC AAG TTC CTG CAC ATC TTT GGC ACC CTG
CTG CCC CGG CCC TCC TTC CTC TAC CGC ACC ATC CAA GAT TTG GGC ATC GGC ACA TTC CGA
GAC TTG GCC GTG GTG CTG GAA ACG GCG CCC ATC CTG ACC GCA CTG GAC ATC TTC GTG GAC
CGG CGT GTG TCT GCG CTG CCT GTG GTC AAC GAA ACT GGA CAG GTA GTG GGC CTC TAC TCT
CGC TTT GAT GTG ATC CAC CTG GCT GCC CAA CAA ACA TAC AAC CAC CTG GAC ATG AAT GTG
GGA GAA GCC CTG AGG CAG CGG ACA CTG TGT CTG GAA GGC GTC CTT TCC TGC CAG CCC CAC
GAG ACC TTG GGG GAA GTC ATT GAC CGG ATT GTC CGG GAA CAG GTG CAC CGC CTG GTG CTC
GTG GAT GAG ACC CAG CAC CTT CTG GGC GTG GTG TCC CTC TCT GAC ATC CTT CAG GCT CTG
GTG CTC AGC CCT GCT GGA ATT GAT GCC CTC GGG GCC TGA GAA CCT TGG AAC CTT TGC TCT
CAG GCC ACC TGG CAC ACC TGG AAG CCA GTG AAG GGA GCC GTG GAC TCA GCT CTC ACT TCC
CCT CAG CCC CAC TTG CTG GTC TGG CTC TTG TTC AGG TAG GCT CCG CCC GGG GCC CCT GGC
CTC AGC ATC AGC CCC TCA GTC TCC CTG GGC ACC CAG ATC TCA GAC TGG GGC ACC CTG AAG
ATG GGA GTG GCC CAG CTT ATA GCT GAG CAG CCT TGT GAA ATC TAC CAG CAT CAA GAC TCA
CTG TGG GAC CAC TGC TTT GTC CCA TTC TCA GCT GAA ATG ATG GAG GGC CTC ATA AGA GGG
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TAG AGC TGC CCT CTC TCA GTT CAG TTC CCC CCT GCT GAG AAT GTC CCT GGA AGG AAG CCA
GTT AAT AAA CCT TGG TTG GAT GGA ATT TGG AGA GTC

Figure 8a

Pig PRKAG3 Splice variant 2

MSFLEQGESR SWPSRAVTTS SERSHGDQGN KASRWTRQED VEEGGPPGPR EGPQSRPVAE
STGQEATFPK ATPLAQAAPL AEVDNPPTER DILPSDCAAS ASDSNTDHLD LGIEFSASAA
SGDELGLVEE KPAPCPSPEV LLPRLGWDDE LQKPGAQVYM HFMQEHTCYD AMATSSKLVI
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Figure 8b

SEQUENCE LISTING

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ANDERSSON, LEIF
LOOFT, CHRISTIAN
KALM, ERNST

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Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr Ser Ser Lys
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Leu Val Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe Phe Ala
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Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp Ser Lys Lys
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cac cgc tat tac agg tcc ccc ctg gtc cag atc tac gag att gaa gaa   717
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cat aag att gag acc tgg agg gag atc tac ctt caa ggc tgc ttc aag   765
His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe Lys
        85                  90                  95

cct ctg gtc tcc atc tct ccc aat gac agc ctg ttc gaa gct gtc tac   813
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Ser Gly Ala Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys Phe
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Leu His Ile Phe Gly Thr Leu Leu Pro Arg Pro Ser Phe Leu Tyr Arg
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Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala Val Val
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Lys Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu
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Glu Glu His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys
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Phe Lys Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala
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Tyr Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala
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Val Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val
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Val Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln
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Thr Tyr Asn His Leu Asp Met Asn Val Gly Glu Ala Leu Arg Gln Arg
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Gly Glu Val Ile Asp Arg Ile Val Arg Glu Gln Val His Arg Leu Val
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Leu Val Asp Glu Thr Gln His Leu Leu Gly Val Val Ser Leu Ser Asp
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cag agc ttt gtg ggg atg ctg acc atc act gac ttc atc ctg gtg ctg   669
Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu Val Leu
                 55                  60                  65

cat cgc tac tac agg tcc ccc ctg gtc cag atc tat gag att gaa caa   717
His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile Glu Gln
             70                  75                  80

cat aag att gag acc tgg agg gag atc tac ctg caa ggc tgc ttc aag   765
His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe Lys
         85                  90                  95

cct ctg gtc tcc atc tct cct aat gat agc ctg ttt gaa gct gtc tac   813
Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val Tyr
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tca ggc aac gta ctc cac atc ctc aca cac aaa cgc ctg ctc aag ttc   909
Ser Gly Asn Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys Phe
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ctg cac atc ttt ggt tcc ctg ctg ccc cgg ccc tcc ttc ctc tac cgc   957
Leu His Ile Phe Gly Ser Leu Leu Pro Arg Pro Ser Phe Leu Tyr Arg
            150                 155                 160

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ctg gag aca gca ccc atc ctg act gca ctg gac atc ttt gtg gac cgg   1053
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cgt gtg tct gca ctg cct gtg gtc aac gaa tgt ggt cag gtc gtg ggc   1101
Arg Val Ser Ala Leu Pro Val Val Asn Glu Cys Gly Gln Val Val Gly
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ctc tat tcc cgc ttt gat gtg att cac ctg gct gcc cag caa acc tac   1149
Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln Thr Tyr
                215                 220                 225

aac cac ctg gac atg agt gtg gga gaa gcc ctg agg cag agg aca cta   1197
Asn His Leu Asp Met Ser Val Gly Glu Ala Leu Arg Gln Arg Thr Leu
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tgt ctg gag gga gtc ctt tcc tgc cag ccc cac gag agc ttg ggg gaa   1245
Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Ser Leu Gly Glu
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gac gag acc cag cat ctc ttg ggc gtg gtc tcc ctc tcc gac atc ctt   1341
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cag gca ctg gtg ctc agc cct gct ggc atc gat gcc ctc ggg gcc tga   1389
Gln Ala Leu Val Leu Ser Pro Ala Gly Ile Asp Ala Leu Gly Ala
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Lys Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu
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Val Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile
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Glu Gln His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys
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